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COMPONENTES BIACTIVOS DEL ALPERUJO: PROPIEDADES Y APLICACIÓN EN FIBRA ALIMENTARIA

TESIS DOCTORAL

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COMPONENTES BIACTIVOS DEL ALPERUJO: PROPIEDADES Y APLICACIÓN EN FIBRA ALIMENTARIA

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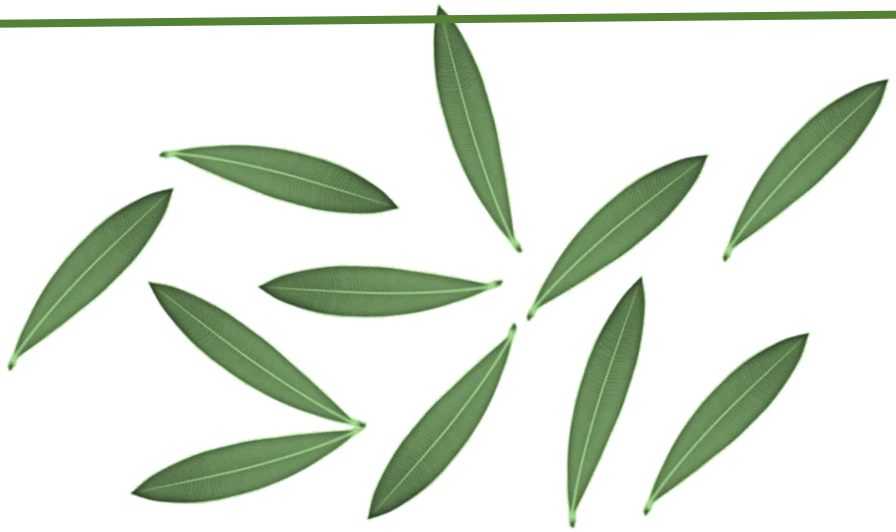


1. Resumen

Los beneficios para la salud de la ingesta de aceite de oliva son de sobra conocidos. De hecho, es la principal fuente de grasas monoinsaturadas de la dieta mediterránea en donde una infinidad de estudios científicos y epidemiológicos han demostrado su incidencia positiva en la prevención de enfermedades cardiovasculares y cáncer. El aceite de oliva es el zumo de la aceituna que a su vez es el fruto de uno de los árboles más longevos y de mayor tradición en la cuenca del mediterráneo, el olivo. Durante muchos siglos el olivo ha supuesto una fuente de salud a través de los productos de la aceituna y de la hoja. Sin embargo, no llegamos a aprovechar todo el potencial que dicho árbol encierra. Y prueba de ello es que las probadas actividades biológicas que presenta el aceite de oliva se deben además de su composición en ácidos grasos a la presencia de componentes minoritarios. Curiosamente tras la extracción del aceite de oliva sólo un 2 % de estos componentes tan interesantes pasan al aceite, quedando el 98 % en el residuo. Actualmente y debido al interés nutricional estos componentes se están empezando a recuperar. Pero siguen existiendo componentes cuyos beneficios para la salud hacen que sea vital su estudio y recuperación.

La industria del aceite de oliva representa uno de los sectores más importantes a nivel productivo en nuestro país, siendo España el mayor productor mundial de aceite de oliva (39% de la producción total) (www.internationaloliveoil.org). La tecnología para la extracción del aceite de oliva ha ido evolucionando en España desde principios de los años sesenta hasta la actualidad, pasando en la mayoría de las almazaras de un sistema de centrifugación de tres fases a uno de dos fases. Sin embargo, esta evolución en el proceso de extracción no ha sido suficiente para evitar la generación de un subproducto con una elevada carga contaminante y un fuerte impacto medioambiental denominado alperujo.

Este subproducto o alperujo, se caracteriza por estar formado por restos de fragmentos de hueso de la aceituna (15% w/w), la pulpa (20% v/v) y agua de vegetación (65% v/v) (**De la Casa y col., 2012**). El alperujo no resuelve los problemas de contaminación que presentaba el sistema de extracción de tres fases y plantea otros nuevos, debido a que

no sólo contiene más humedad que el orujo de tres fases, sino que también posee una elevada carga contaminante debido a su pH ácido, su elevada salinidad tóxica y a que exhibe valores altos de electroconductividad. Además, presenta un alto contenido en sustancias orgánicas, que incluyen carbohidratos, polifenoles, lípidos y proteínas.

La gestión y eliminación de los enormes volúmenes generados de este subproducto a partir de la producción del aceite de oliva presentan un gran problema de polución medioambiental que permanece todavía sin resolver. Sin embargo, este subproducto presenta una alta concentración en productos de alto valor, tales como polifenoles y pectinas.

Si se tiene presente la implicación del estrés oxidativo en los procesos de inflamación, los abundantes compuestos antioxidantes presentes en el aceite de oliva pueden aportar numerosos beneficios en enfermedades inflamatorias. El hidroxitirosol (HT) es un fenol simple y se considera que es el principal agente antioxidante presente en el aceite de oliva con una potente actividad antiinflamatoria, mientras que el 3,4-dihidroxifenilglicol (DHFG) es otro fenol simple presente en la fruta de olivo con la misma estructura orto-difenólica que la HT pero con un grupo hidroxilo adicional en posición β. DHFG presenta una alta capacidad antioxidante por lo que se podría pensar en una potencial capacidad inflamatorias. A persar del potencial biológico de estos fenoles simples es necesario mejorar sus propiedades funcionales y su biodisponibilidad. Para ello, en este trabajo se estudió la interacción pectina-HT, pectina-DHFG mediante su encapsulación en perlas de pectinato de calcio. Los resultados muestran que los encapsulados pueden retener los compuestos hidrosolubles HT y DHFG en cantidad suficiente para su efecto en el colón. También mostraron que después de una digestión gástrica e intestinal simulada, las encapsulaciones pueden retener los compuestos hidrosolubles HT y DHFG en cantidad suficiente para su posible efecto in-situ en el colón y su posible beneficio en enfermedades inflamatorias intestinales. Debido a que esta cantidad encapsulada era prácticamente imposible recuperarla, se sugirió la formación de un fuerte complejo pectina-HT y pectina DHFG, el cual presenta actividad antioxidante lo que podría promover la salud intestinal.

Así mismo, teniendo presente los efectos beneficiosos que el HT posee y la posible interacción HT-pectina, se han realizado ensayos *in vivo* en modelo animal (ratas). La enfermedad inflamatoria intestinal (EII) abarca varias afecciones inflamatorias crónicas, las más significativas son la colitis ulcerosa (CU) y la enfermedad de Crohn (EC). No se conoce ningún tratamiento que consiga curar por completo la EII, actualmente existen desde tratamientos de terapia farmacológica hasta métodos quirúrgicos, consiguiendo únicamente el control y reducción de las molestias que conlleva dicha enfermedad. Estas molestias disminuyen significativamente la calidad de vida de los pacientes, así como las cirugías, las cuales implican morbimortalidad. En función a las propiedades que conocemos del hidroxitirosol, el objetivo de este estudio fue mejorar la disponibilidad del HT en el colon en donde pueda hacer efectiva dichas propiedades, como el antiinflamatorio ayudando a la remisión a largo plazo de la enfermedad, lo que conllevaría, una mejora en la calidad de vida. Por tanto, se realizaron formulaciones pectina/alginato y aceite de oliva emulsionadas con hidroxitirosol para evaluar la efectividad del HT en la EII. Los resultados sugieren que el grupo de ratas tratadas con la emulsión que presentaba HT, disminuye la colitis ulcerosa, demostrando así su efecto antinflamatorio y, por tanto, su efecto en el tratamiento de la enfermedad de inflamación intestinal.

Por otro lado, las propiedades antioxidantes y antibacterianas que el HT y DHFG presentan nos permitió su aplicación en alimentos perecederos. Las enfermedades postcosecha de frutas y verduras son un problema importante en el almacenamiento de productos. En concreto las fresas es una fruta que se consume en todo el mundo presentando una vida media corta. Es un alimento que se deteriora fácilmente debido a los daños mecánicos, la pérdida de agua, y la aparición de hongos. La formulación de un recubrimiento comestible en base de pectina/proteína de pescado (gelatina) al cual se le fue añadido HT y DHFG para comprobar su eficacia como agente antifúngico. Los resultados mostraron que aquellos recubrimientos que presentaban HT/DHFG en su formulación retrasaron significativamente la aparición de hongos en las fresas. Además, la oxidación de los lípidos es el principal factor que contribuye al deterioro de la carne cruda durante el almacenamiento, causando una reducción de la calidad y aceptabilidad debido al desarrollo

de un sabor y rancidez indeseables. Es por ello que se formuló una película comestible de pectina/ proteína de pescado (gelatina) con o sin cera de abeja para prevenir la oxidación de los lípidos sobre carne de ternera almacenada a 4ºC. Los resultados experimentales mostraron que las muestras de carne envueltas con una película que contenía antioxidantes redujeron la formación de productos de oxidación en forma de sustancias de reacción del ácido tiobarbitúrico (TBARS) en comparación con la película de control sin antioxidantes

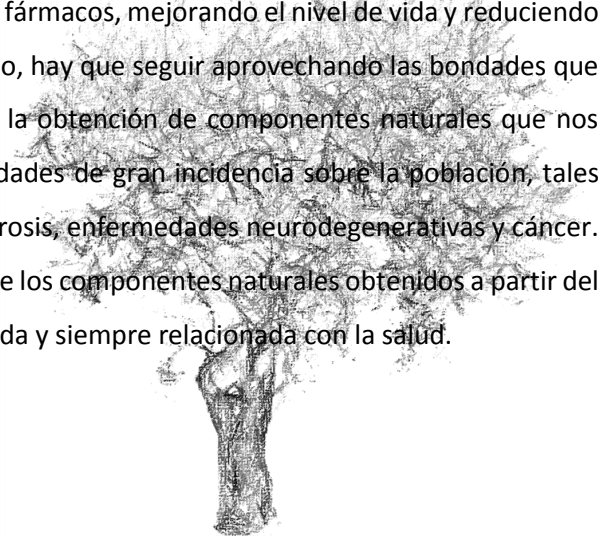
La fibra alimentaría es una parte esencial de nuestra dieta, y se caracteriza porque pasa el tracto intestinal sin ser digeridas por las enzimas humanas. Únicamente son parcialmente fermentados por las bacterias del colon. Otro de los estudios realizados fue estudiar la interacción de la pared celular de manzana y fresa con los compuestos fenólicos HT y DHFG. Después de digestiones simuladas *in vitro* complejos de HT/DHFG fibras solubles e insolubles fueron obtenidas y estudiadas. La fibra obtenida después de las digestiones simuladas retenía una cantidad considerable de los compuestos antioxidantes. De hecho, estas propiedades antioxidantes seguían presentes después de la formación del complejo. Obteniendo una fibra funcionalizada, permitiendo combinar las propiedades saludables tanto de la fibra como de los compuestos fenólicos.

EL alperujo es un subproducto de naturaleza vegetal, y se encuentra enriquecido en los diferentes componentes de la pared celular de la aceituna. La pared celular está compuesta por polisacáridos complejos cuya naturaleza puede ser neutra (hemicelulosas y celulosas) o ácida (pectinas). Entre las pectinas éstan las denominadas pectinas modificadas (PM) con interesantes propiedades biológicas. El término PM incluye pectinas con distinta composición y características comunes. Son fragmentos de bajo Pm que en teoría pueden absorberse, algunas de las cuales pueden unirse y bloquear la proteína oncogénica galectina-3 (Gal-3). Actualmente, la mayoría de las investigaciones realizadas con PM emplean pectinas obtenidas a partir de cítricos (MCP). Las pectinas utilizadas en nuestros estudios fueron obtenidas a partir del alperujo mediante tratamiento con vapor directo y con diferentes post-tratamientos, obteniéndose diferentes extractos ricos en pectinas modificadas, así como ricos en polifenoles.

Se han aislado y caracterizado extractos ricos en polifenoles asociados a polisacáridos de bajo peso molecular, ricos en pectinas, del alperujo mediante tratamiento térmico con vapor directo. Estos extractos exhibieron una fuerte actividad antioxidante ORAC, así como un fuerte efecto en la proliferación de células de carcinoma de colon (Caco-2), líneas celulares de leucemia monocítica (THP-1), así como cuatro líneas de cáncer vesical (RT112, T24 J82 y SCaBER). Además, para el caso de las líneas de cáncer vesical, los efectos proliferativos de los extractos obtenidos del alperujo fueron comparados con un agente quimioterapéutico conocido como el cisplatino (CDDP), obteniendo resultados similares a éste, así, como mejores resultados antiproliferativos si se compara con la pectina comercial.

Todos los extractos exhibieron fuertes efectos antiproliferativos y antioxidantes, lo que podría ser debido al elevado contenido en polifenoles. Por tanto, se realizó un estudio donde los polifenoles eran eliminados con el fin de observar el efecto y observar que efectos antioxidantes y antiproliferativos presentaban dichos extractos blanqueados sobre líneas de Caco-2 y THP-1, los cuales presentaban valores proliferativos similares a la pectina modificada comercial, lo que verifica de manifiesto la importancia en nuestros extractos de los polifenoles asociados.

Todo ello pone de manifiesto que el uso de componentes naturales es uno de los principales objetivos en el campo de la alimentación y la salud, tanto para disminuir o eliminar el uso de sustancias sintéticas y fármacos, mejorando el nivel de vida y reduciendo la incidencia de enfermedades. Por tanto, hay que seguir aprovechando las bondades que nos suministra el olivo y traducirlas en la obtención de componentes naturales que nos ayuden a combatir y prevenir enfermedades de gran incidencia sobre la población, tales como; diabetes, hipertensión, aterosclerosis, enfermedades neurodegenerativas y cáncer. Se observa la gran importancia del uso de los componentes naturales obtenidos a partir del olivo como una fuente ancestral vinculada y siempre relacionada con la salud.



2. Introducción

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2.1 El Alperujo

La industria del aceite de oliva representa uno de los sectores más importantes a nivel productivo en nuestro país, siendo España el mayor productor mundial de aceite de oliva (38% de la producción total, (2017/2018) (www.internationaloliveoil.org). La tecnología para la extracción del aceite de oliva ha ido evolucionando en España desde principios de los años sesenta hasta la actualidad, pasando en la mayoría de las almazaras de un sistema de centrifugación en continuo de tres fases a uno continuo de dos fases (**Fig.1**). Sin embargo, esta evolución en el proceso de extracción no ha sido suficiente para evitar la generación de un subproducto con una elevada carga contaminante y un fuerte impacto medioambiental denominado alperujo.

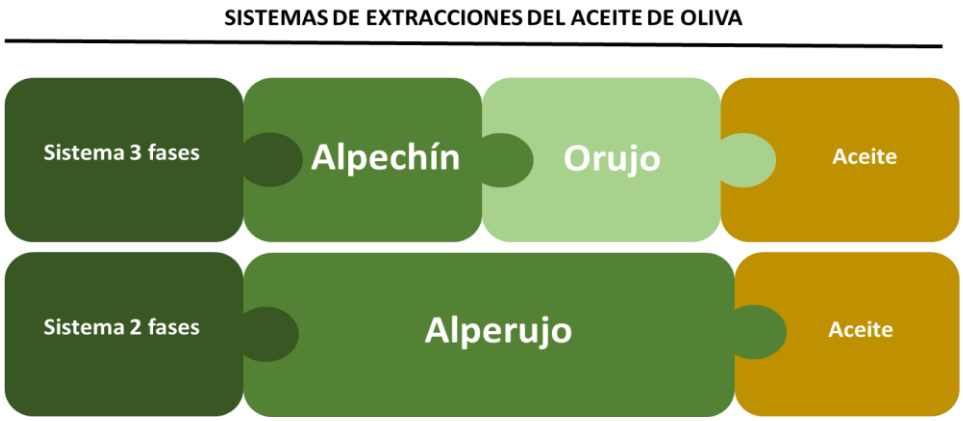

Figura 1. Sistema de extracciones de dos y tres fases del aceite de oliva.

El alperujo es una pasta semisólida o viscosa que resulta de la producción de aceite de oliva en dos fases, siendo este uno de los principales subproductos generados en la industria agroalimentaria, de hecho, en España se genera más de 5 millones de toneladas por campaña. Es una mezcla compleja formada por piel, pulpa y huesos, la cual presenta una elevada humedad (70%). Su composición incluye además de una fracción mineral, un alto contenido en materia orgánica (superior al 90%), con abundantes cantidades de celulosa, hemicelulosas y pectinas además de niveles importantes de lípidos, glúcidos y fenoles (**Nunes y col., 2016**). Éste presenta un alto contenido en

fenoles y otros muchos componentes minoritarios de la aceituna, se considera hoy una materia prima valiosa rica en componentes activos de interés, de naturaleza y composición variadas, que pueden extraerse y utilizarse para las industrias alimentaria, farmacéutica y cosmética (**Roselló-Soto y col., 2015a; Yener 2015; Nunes y col., 2016; Rodrigues y col., 2017**)

2.1.2 Compuestos de alto valor añadido.

Las elevadas cantidades de alperujo que producen las industrias del aceite de oliva, pueden ser usadas para la recuperación de compuestos o sustancias valiosas y la conversión en productos útiles mediante el desarrollo de nuevos procesos. Esto sería de gran relevancia no solo desde el punto de vista medioambiental y económico, sino también para diferentes sectores de la industria (alimentaría, cosmética y farmacéutica) en los que dichos compuestos pondrían tener aplicación. El alperujo presenta un alto contenido en compuestos valiosos, como fenoles y polisacáridos. La elevada carga contaminante que el alperujo tiene es debida al alto contenido de fenoles (efecto fitotóxico) (**Malapert y col., 2018**). Sin embargo, si estos fenoles son recuperados, pueden tener actividades muy importantes, tales como; antioxidantes, antiinflamatorios, antibacterianos y anticancerosos (**Parkinson, & Cicerale, 2016**). Curiosamente tras la extracción del aceite de oliva sólo un 2 % de estos componentes tan interesantes pasan al aceite, quedando el 98 % en el subproducto, siendo principalmente hidroxitirosol y sus derivados. (**Reboredo-Rodríguez y col., 2017**). Por lo tanto, la extracción de estos fenoles como productos de alto valor añadido podría ser considerada como una interesante alternativa natural y barata para hacer provechosos los residuos de las almazaras.

2.1.2.1 Polifenoles

Los polifenoles presentes en el alperujo han demostrado poseer actividad antioxidante, antimicrobiana y antifúngica. Algunos de los antioxidantes comerciales encontrados en el alperujo son el tirosol (Ty), el hidroxitirosol (HT), 3-4 dihidroxifenilglicol (DHFG) y en menor concentración la oleuropeína, los cuales pueden ser usados como aditivos alimentarios o cosméticos. Durante los últimos años el interés por la

recuperación de los compuestos fenólicos desde los subproductos de la industria del aceite, tanto desde los generados desde el sistema de tres fases (**Obied y col., 2008; Dermeche y col., 2013; Kalogerakis y col., 2013**), como los generados con el sistema de dos fases (**Lesage-Meessen y col., 2001; Fernández-Bolaños y col., 2006**) ha aumentado drásticamente. Un gran número de artículos relacionan los efectos beneficiosos del aceite de oliva con su contenido fenólico (**Ghanbari y col., 2012; Buckland, & Gonzalez, 2015**). De hecho, el consumo de aceite de oliva y aceitunas de mesa ha mostrado estar asociado con bastantes efectos saludables, incluyendo una disminución en la incidencia de enfermedades cardiovasculares y ciertos tipos de cáncer (**Ruiz-Canela y col., 2014; Buckland, & Gonzalez, 2015; Toteda y col., 2017**). La recuperación de estos compuestos a partir del alperujo puede cambiar la visión negativa que se tiene de este subproducto, y reconocerlo como una fuente barata y una valiosa materia prima para la producción de compuestos bioactivos naturales con un amplio espectro de actividades biológicas **(Cicerale y col., 2010**).

2.1.2.2 Polisacáridos

Por otro lado, el alperujo al ser un residuo de naturaleza vegetal también se encuentra enriquecido en los diferentes componentes de la pared celular de la aceituna. La pared celular está compuesta por polisacáridos complejos cuya naturaleza puede ser neutra (hemicelulosas y celulosas) o ácida (pectinas). Recientes estudios han mostrado que una parte sustancial de las hemicelulosas pueden ser recuperadas en forma de oligosacáridos mediante un tratamiento auto-hidrolítico desde la fracción soluble en agua del alperujo (**Rodríguez y col., 2007a**). **Lama-Muñoz y col.** (2012) aíslan a partir del alperujo tratado térmicamente previa extracción de los fenoles con acetato de etilo e hidrólisis suave una serie de oligosacáridos entre los que destacan xiloglucanos y xilo-oligosacáridos. **Hsu y col**. (2004) observan que los xilooligosacáridos usados como suplementos dietéticos pueden ser beneficiosos para la salud gastrointestinal, y reducen el riesgo de cáncer. Además, los oligosacáridos con grado de polimerización comprendidos entre 3 y 10 unidades se caracterizan por ser prebióticos, favoreciendo el

crecimiento selectivo y/o actividad de un número limitado de bacterias del colon, presentando importantes propiedades fisiológicas beneficiosas para la salud de los consumidores (**Gibson & Roberfroid, 1995**). Es por esta razón que su uso como ingredientes en alimentos funcionales se ha visto incrementado (**Mussatto & Mancilha, 2007**).

Galanakis y col. (**2010**) también obtuvieron una fracción soluble de polisacárido concentrando térmicamente seguida de un proceso de precipitación de ácido cítrico-etanol, el cual fue capaz de formar geles, a pesar de la baja concentración de pectina y el alto grado de metilación (59%) de los polisacáridos pécticos. Así mismo, diferentes autores han sido capaces de aislar pectinas ricas en arabinosas a partir del alperujo (**Cardoso y col., 2003; Coimbra y col., 2010**). Comercialmente las pectinas se extraen de dos importantes fuentes, del limón y la manzana. Pero en los últimos años ha aumentado el interés por buscar nuevas fuentes de pectinas, empleando para ello residuos de naturaleza vegetal. Las pectinas se caracterizan porque se pueden emplear como agentes gelificantes, emulsificantes y estabilizantes en la industria alimentaria. Pueden actuar como prebióticos, antiinflamatorios, antidiarreicos, controlan la diabetes y previenen numerosas enfermedades como obesidad y cáncer (**Rodríguez y col., 2006; Morris y col., 2013; Olano-Martin y col., 2003; Maxwell y col., 2012; Samuelsson y col., 2016**). Además de ser capaces de unirse a ácidos biliares causando su eliminación en las heces y disminuyendo por tanto los valores de colesterol (**Eastwood & Halminton, 1968**). **Rubio-Senent y col**., (2015 a, b) obtiene del alperujo polisacáridos pécticos con importantes propiedades tales como retención de ácidos biliares, y retardo del índice de la glucosa, además de actividad antioxidante debido a la vinculación con compuestos fenólicos.

2.2 Recuperación de compuestos de interés.

2.2.1. Tratamientos.

Para aprovechar los principales componentes del alperujo (celulosa, hemicelulosas, pectinas y polifenoles) se requiere un paso previo para que se rompan las barreras físicas y químicas que este material presenta ya que existe una íntima

interacción entre la celulosa, hemicelulosas, pectinas y polifenoles. Esta interacción condiciona la recuperación de los compuestos de interés, de ahí la necesidad de un pretratamiento el cual posibilita la separación de las fases (líquida y sólida) y provoca la solubilización de los compuestos de interés, como son los compuestos fenólicos y azúcares, permitiendo así su recuperación. Existen numerosos sistemas de tratamientos para recuperar los compuestos de interés, aunque la eficiencia de recuperación dependerá de la tecnología utilizada.

2.2.2. Tratamiento de explosión al vapor o "steam explosion" (SE).

La viabilidad del aprovechamiento y utilización de los subproductos de la extracción del aceite de oliva, como de otros subproductos de origen vegetal, y del alperujo en particular, pasa por encontrar pretratamientos industriales viables y económicamente rentables para alcanzar una eficiente recuperación de sus componentes.

Para aprovechar los principales componentes del alperujo y los de alto valor añadido se requiere un paso previo para romper las barreras físicas y químicas de su complejo entramado estructural. Existen diferentes métodos de pre-tratamiento, entre los que se encuentra el pre-tratamiento térmico con vapor de agua a alta presión y temperatura, con o descompresión rápida (explosiva), "steam explosion".

En esta línea, el grupo de Fitoquímicos, biactividad y desarrollo de procesos del Instituto de la Grasa (C.S.I.C.) trabaja desde hace algunos años en el estudio del aprovechamiento de los subproductos de extracción del aceite de oliva. Para este objetivo usan vapor de agua a alta presión mediante un tratamiento de explosión al vapor que facilita la extracción de compuestos de alto valor añadido, debido a que por esta técnica se solubiliza gran parte de los compuestos fenólicos presentes en la matriz del alperujo (**Fig.2**) (**Fernández-Bolaños y col., 2001; Rodríguez y col., 2007b**). Este tratamiento se ha usado para la solubilización de compuestos fenólicos con actividad antioxidante a partir de orujo de aceituna de dos y tres fases, permitiendo la

recuperación de HT y otros compuestos de interés (**Fernández-Bolaños y col., 1998; Felizón y col., 2000., Fernández-Bolaños y col., 2002a**).

El fundamento del tratamiento SE sobre biomasa lignocelulósica consiste en tratarla con vapor saturado a alta presión seguido de una rápida despresurización, lo que provoca que el material sufra una descompresión explosiva. En este tratamiento el proceso se lleva a cabo a temperaturas dentro del intervalo de 160-240 °C (correspondiente a presiones comprendidas entre 0,69-4,46 MPa) durante cortos periodos de tiempo, desde varios segundos a pocos minutos antes de que el material sea expuesto a presión atmosférica (**Duff y Murria, 1996**). El efecto del SE sobre el material es una combinación de modificaciones físicas y químicas.

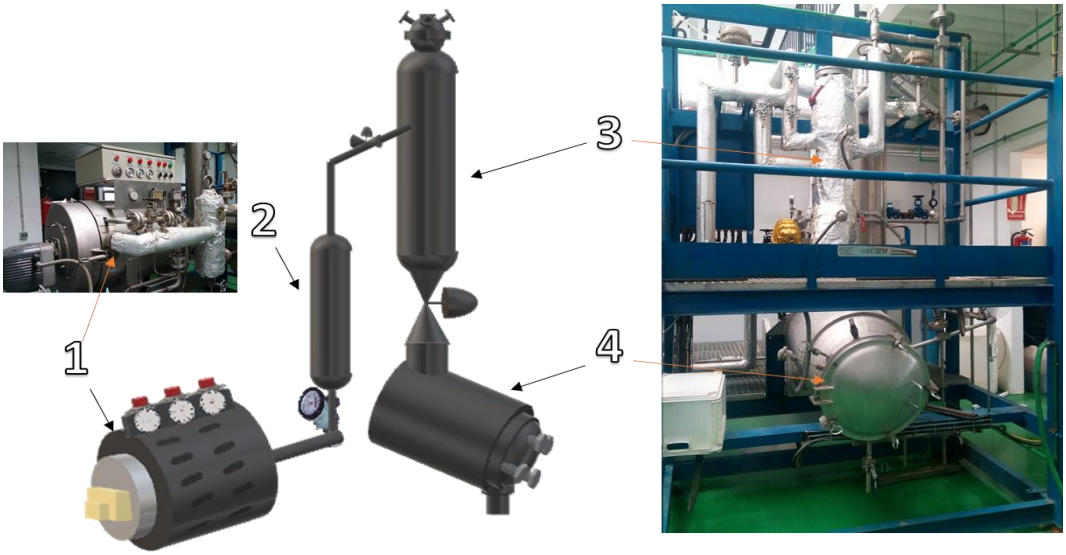

Figura 2. Representación esquemática y fotografía del reactor de explosión al vapor. En el esquema pueden verse los componentes principales del reactor: (1) caldera de producción de vapor, (2) acumulador de vapor, (3) cámara de reacción y (4) depósito de expansión.

Las modificaciones físicas se producen como consecuencia de la rápida despresurización, provocando en las regiones más débiles (celulosa amorfa) un resquebrajamiento de la estructura lignocelulósica y la separación de las fibras de celulosa, como consecuencia se produce una reducción del tamaño de partícula y la expansión de los microporos. Las condiciones de presión y temperatura provocan la

disociación del agua generando iones hidronio, que junto a los compuestos formados in situ, entre los que destaca el ácido acético procedente de la desacetilación de las hemicelulosas, dan lugar a un medio ácido (pH 3-4), y con ello a la acción catalítica en la autohidrólisis (**Duff & Murria, 1996**), que desencadenan las modificaciones químicas. De esta forma los grupos ácidos catalizan la hidrólisis de las hemicelulosas, favorecen aún más la disminución en el tamaño de los cristales de la celulosa (**Mok & Antal, 1992**) y se produce una ruptura de los enlaces hemicelulosa-lignina facilitando el acceso de las enzimas a las fibras de celulosa (**Mosier y col., 2005**). Las elevadas temperaturas alcanzadas en el reactor provocan que la lignina se funda rompiéndose las uniones con los demás componentes de la pared celular, al bajar la temperatura la lignina liberada condensa formando gotas (**Fig. 3**). Aunque el ácido acético se ha considerado el principal ácido responsable del efecto catalítico de la autohidrólisis, también se producen otros ácidos como el ácido fórmico y el levulínico que ayudan a la bajada del pH (**Ramos, 2003**).

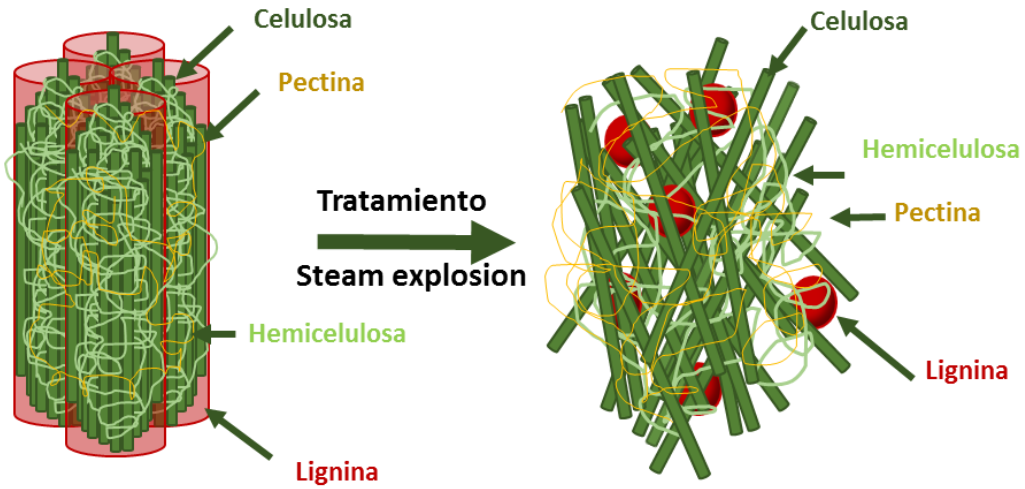

Figura 3. Representación esquemática del efecto del pretratamiento "stean explotion" sobre materiales lignocelulósicos

Comparado con los métodos de tratamiento alternativos, como los procesos de hidrólisis ácida y los procesos oxidativos, el tratamiento SE aplicado al alperujo destaca debido a que reduce considerablemente el impacto ambiental, los costes económicos y el consumo energético. Además, elimina el uso de cantidades significativas de productos

químicos ácidos y/o básicos (Ramos, 2003) y es posible una recuperación más completa de todos los biopolímeros (celulosa, hemicelulosas, lignina) en una forma útil, así como recuperar compuestos fenólicos de interés, tales como el HT y el Ty, al provocar su solubilización (**Fernández-Bolaños y col., 1998**).

2.2.3 Tratamiento térmico con vapor

A pesar de las ventajas que proporciona en el caso del alperujo el pretratamiento con vapor y descompresión explosiva, aparecen inconvenientes para su puesta en práctica a nivel industrial. El mayor inconveniente se debe a las condiciones de altas temperaturas (160-240 ºC) y presiones (máxima 4,46 MPa) que son necesarias, seguida de una rápida descompresión, que limita el desarrollo de este sistema en las instalaciones de las industrias orujeras que se encargan de la recepción y del tratamiento del alperujo para extraer el aceite de orujo, y las cuales trabajan a presiones máximas de 0,88 MPa. Por este hecho nuestro grupo de Investigación (Fitoquímicos, biactividad y desarrollo de procesos del Instituto de la Grasa, C.S.I.C.) ha diseñado, construido y puesto en marcha a escala planta piloto un nuevo sistema de tratamiento térmico con vapor discontinuo que funciona a temperaturas y presiones más bajas y sin necesidad de descompresión explosiva. Con este nuevo reactor se pretende mantener las ventajas anteriormente mencionadas para el aprovechamiento del alperujo y reducir la temperatura (máxima 190 ºC) y la presión (máxima 1,18 MPa) eliminando factores críticos como la presencia de oxígeno, gracias a la posibilidad de generar vacío en su interior y la descompresión rápida (**Fernández-Bolaños y col., 2012**).

Estudios realizados con el nuevo reactor demuestra que éste proporciona las mismas ventajas sobre el alperujo que el reactor de SE permitiendo adaptar su uso más fácilmente a la industria orujera (**Lama- Muñoz y col., 2011**).

Figura 4. Representación esquemática y fotografía del reactor del nuevo reactor de tratamiento térmico. En el esquema pueden verse los componentes principales del reactor: (1) caldera de producción de vapor, (2) acumulador de vapor, (3) cámara de reacción.

Es un reactor en discontinuo de 100 L de capacidad, de construcción cilíndrico vertical y material de acero inoxidable INOX-316L. (**Fig.4**) Permite el tratamiento del alperujo con vapor en condiciones menos severas y oxidantes que las que se alcanzan en el anterior reactor con vapor y descompresión explosiva ("steam explosion"). El proceso térmico con vapor de agua favorece la solubilización de parte de las hemicelulosas y la liberación de ácidos que a su vez favorecen la autohidrólisis de la misma. Por lo tanto, el tratamiento térmico con vapor hidroliza parcialmente al alperujo liberando algunos enlaces glicosídicos y fragmentando biopolímeros, lo que facilita la disociación de sus componentes y una eficaz separación sólido-líquido. El tratamiento también solubiliza a una gran cantidad de compuestos bioactivos de interés, quedando un sólido reducido en el que se concentra la grasa. En el aceite extraído a partir del alperujo tratado se produce un considerable incremento en las concentraciones de una serie de componentes minoritarios (esteroles, tocoferoles, alcoholes triterpénicos, etc) de gran interés para la salud (**Lama-Muñoz y col., 2011)**. En el caso de la fracción sólida se consigue una

reducción drástica de la humedad de más del 20%, obteniéndose un sólido con solo el 30% de humedad. Este hecho, de enorme importancia económica por el ahorro energético en los secaderos, favorece la extracción del aceite y el posterior uso de la biomasa como biocombustible. Además, en la fracción líquida, también se solubiliza una parte muy importante de los compuestos fenólicos de gran interés, así como carbohidratos en forma de mono-, oligo- y polisacáridos (**Lama-Muñoz y col., 2012**).

2.3. Compuestos fenólicos en la aceituna.

Los fenoles son un grupo heterogéneo de compuestos naturales presentes en todas las plantas superiores, que poseen la característica química común de poseer al menos un grupo fenol. Entre ellas, el olivo y su fruto han sido reconocidos como una extraordinaria fuente de fenoles. En las plantas, los compuestos fenólicos son metabolitos secundarios y desempeñan funciones fisiológicas: actúan como agentes que reducen el crecimiento de plantas competidoras, por su carácter antimicrobiano son productos de defensa ante microorganismos patógenos como bacterias, hongos y virus, y absorben la radiación ultravioleta en las capas más superficiales protegiendo a los tejidos externos de sus efectos perjudiciales (**Ryan & Robards, 1998**). Estos compuestos están presentes en todas las diferentes partes de la planta, pero su naturaleza y concentración varía entre los diferentes tejidos (**Covas & col., 2006**).

La composición media de las aceitunas es de un 50% de agua, 1.6% de proteínas, 22% de aceite, 19.1% de carbohidratos, 1.5% de sustancias inorgánicas y de un 1 a un 3% de compuestos fenólicos (**Ghanbari y col., 2012**). Esta composición fenólica es característica y la existencia y cantidad de fenoles específicos en la aceituna, y, por tanto, en el alperujo, dependen de la variedad y estado de madurez, condiciones climáticas, estacionales y geográficas (**Bianchi, 2003**).

Durante el proceso de extracción del aceite de oliva, los fenoles presentes en el fruto se reparten entre la fracción lipídica (aceite de oliva) y los subproductos generados (alpechín y orujo para el sistema de extracción de tres fases, o alperujo en el sistema de extracción de dos fases), en función a su naturaleza hidrofílica o lipofílica. Por lo tanto,

los fenoles presentes en el fruto quedan repartidos entre ambas fracciones, en función de sus constantes de reparto (**Artajo y col., 2007**). La mayoría de los fenoles tienen bajos coeficientes de reparto (aceite/agua) comprendidos entre $6 \cdot 10^{-4}$ y 1,5 (**Rodis y col., 2002**) por lo que se distribuyen preferentemente en la fase acuosa (**Obied y col., 2005**), de forma que sólo el 2% de los fenoles presentes en el fruto pasan a formar parte del aceite de oliva, permaneciendo el 98% restante en el subproducto generado.

Por lo tanto, el contenido fenólico está estrechamente relacionado con el proceso de extracción, el cual determina el comportamiento de los fenoles y por tanto su distribución entre el aceite y las fracciones residuales. Se han identificado alcoholes fenólicos en los que hay que destacar hidroxitirosol (HT) y el tirosol (Ty) los cuales se encuentran en mayor proporción, además del 3,4- dihidroxifenilglicol (DHFG). En el grupo de los fenoles ácidos se han identificado especies como los ácidos, cafeico, protocatéquico, y ferúlico, (**Ghanbari y col., 2012; Ryan & Robards, 1998**). La oleuropeína, un éster glucosídico de 3,4-dihidroxifeniletanol (hidroxitirosol) y ácido elenólico, es el principal compuesto secoiridoide en aceitunas verdes/no maduras; su concentración disminuye con la maduración del fruto. En paralelo con la disminución de la oleuropeína hay un aumento de la desmetiloleuropeína y de la forma dialdehídica del aglicón de la oleuropeína descarboxilmetilada (3,4-DHPEA-DEA) (**Romero y col., 2004**). Esta última sustancia llega a ser predominante en aceitunas maduras. Otros compuestos secoiridoides encontrados en aceitunas son el glucósido de ácido elenólico, el ligustrósido y la forma dialdehídica de su aglicón (**Obied y col., 2007**). Entre los compuestos fenólicos que también han sido identificados en la aceituna se encuentran el verbascósido (y el salidrósido (glucósido de tirosol), y en concentraciones relativamente altas de flavonoides como apigenín-7-O-glucósido, luteolín-7-O-glucósido (**Romero y col., 2002**) (**Fig.5**) Por lo tanto, el perfil fenólico del alperujo es complejo y hasta la fecha la recuperación de éstos compuestos no ha sido completamente llevada a cabo para la valorización del subproducto.

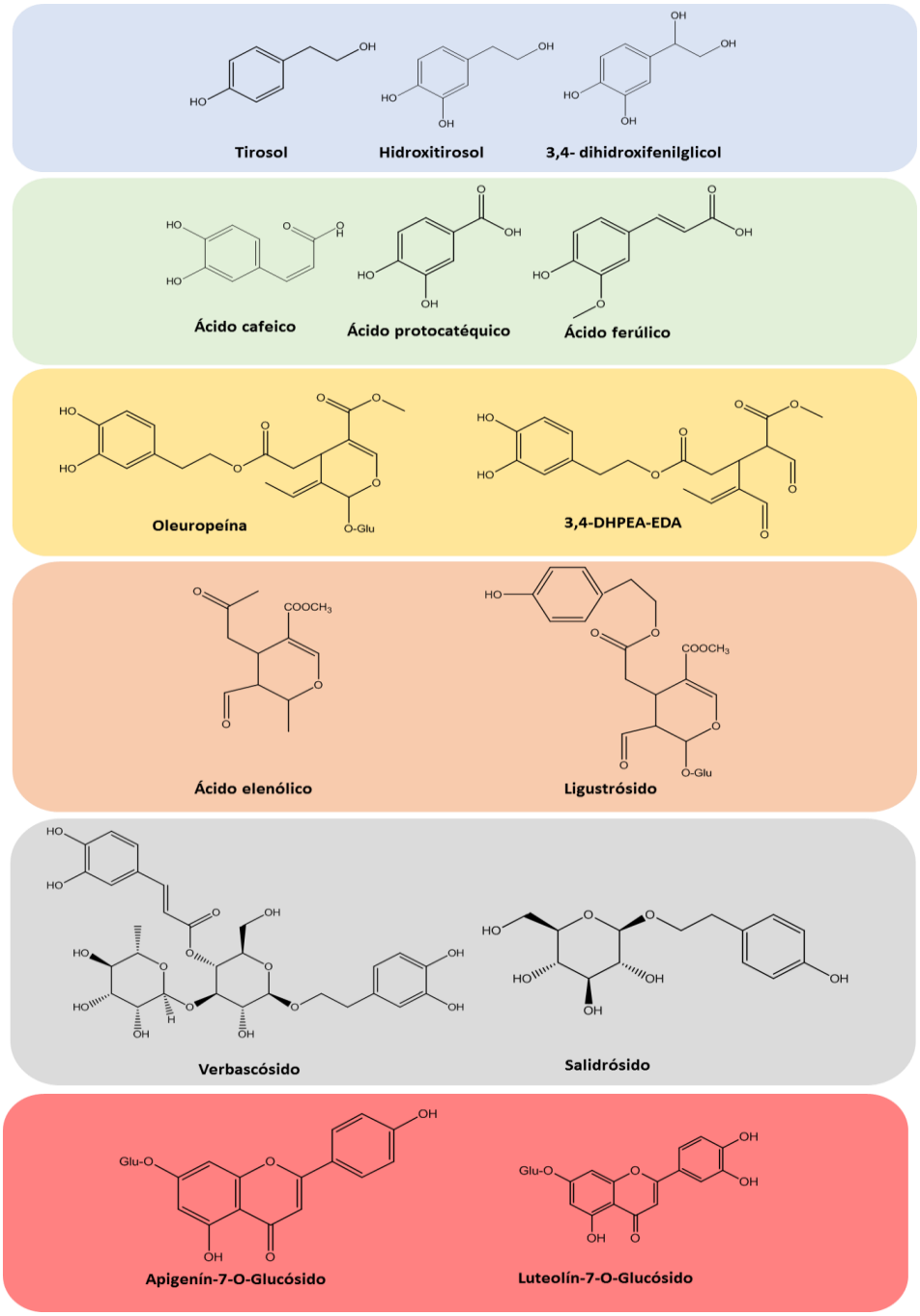

Figura 5. Estructura de los principales compuestos fenólicos

2.3.1 Propiedades de los compuestos fenólicos.

Estudios epidemiológicos indican que la dieta mediterránea está asociada a una menor incidencia de enfermedades cardiovasculares, ateroesclerosis y ciertos tipos de cánceres (**Casas y col., 2014**). Se trata de una dieta rica en frutas y vegetales frescos, con baja proporción de grasa animal, y teniendo al aceite de oliva como la principal fuente de materia grasa. Estos efectos beneficiosos se atribuyen no solo a la baja proporción en ácidos grasos saturados en relación a monoiinsaturados del aceite de oliva, sino también a la presencia de los denominados componentes minoritarios y, en particular, a los compuestos fenólicos antioxidantes (**Pérez-Jiménez y col., 2007**). Las propiedades reconocidas de los polifenoles presente en la aceituna hacen de ellos candidatos ideales para su uso como agente antioxidante, de ahí el interés en la obtención de dichos compuestos.

2.3.2 Hidroxitirosol (HT)

El fenil etil alcohol, 2-(3,4-dihidroxifenil)-etanol, conocido como hidroxitirosol (HT), que procede principalmente de la hidrólisis de oleuropeína, es un glucósido amargo que puede llegar a constituir más del 14% del peso seco del fruto del olivo. El HT es el alcohol fenólico más abundante en la aceituna, en forma libre o conjugada (**Fabiani y col., 2002; Visioli y col., 2004**). Su estructura orto-difenólica, característica de los biofenoles, le confiere una alta capacidad antioxidante, dicha actividad se ha demostrado ser más efectiva que la del antioxidante sintético butilhidroxitolueno (BHT) o la de la vitamina E (**Aruoma, 1998a**), ambos usados ampliamente en la industria alimentaria. Además de actividades antioxidantes el HT muestra una amplia gama de efectos beneficios para la salud, cardiovasculares, anticancerígenos, y efectos antiinflamatorios (**Zhang y col., 2009; Bernini, y col., 2013; Toteda y col., 2017**).

Sin embargo, sus propiedades farmacocinéticas son desfavorables, ya que compuesto muestra baja biodisponibilidad oral y rápida eliminación en humanos (**Robles-Almazán y col., 2018**). Estas características y su carácter hidrofílico limita su uso como un suplemento dietético, así como un aditivo en alimentos (**Mateos y col., 2016;**

Navarro & Morales, 2015). La absorción del HT tiene lugar en el intestino delgado, presentando una rápida absorción que alcanza a los 5-10 minutos la máxima concentración plasmática. Se localiza mayoritariamente en el riñón, siendo este su principal vía de eliminación, aunque también se puede localizar en hígado y cerebro. El tiempo máximo de eliminación del HT y sus metabolitos es de unas 6 horas en humanos. (**Echevarría y col., 2017**).

Debido a las numerosas propiedades que presenta el HT y al elevado interés en este compuesto, numerosos estudios han sido realizados para comprobar el efecto tóxico que podría presentar. Los estudios realizados determinan que no existe efecto tóxico, tanto *in- vivo* como *in- vitro*. Un estudio realizado por **Christian y col.** (2004) con HIDROX ™ (un extracto acuoso, con un contenido de HT alrededor del 70%), el cual se le administraba una dosis de 2g/kg administrado por sonda oral durante 90 días en ratas Sprague Dawley y ratones CD1 ICR (BR) observandose una mortalidad y morbilidad nula. **Aunon-Calles y col.** (2013a) realizaron un estudio más exhaustivo para evaluar los efectos toxicológicos del HT puro. Dosis de 5, 50 y 500 mg / kg / día durante 13 días no dio lugar a alteraciones micro y macroscópicas ni a la muerte. Se observó una ganancia en el peso corporal y un mayor peso renal en el grupo con la dosis de tratamiento más alta, sin cambios en la funcionalidad o estructura del órgano. Además, **Aunon-Calles, y col.** (2013b) determinaron en modelos *in vitro* que el HT en condiciones fisiológicas, no presentaba ningún signo de efectos genotóxicos y mutagénicos.

De hecho, la EFSA (European Food Safety Authority - EFSA) informó que los polifenoles presentes en el aceite de oliva ofrecen protección frente al daño oxidativo. Se ha demostrado que el daño oxidativo afecta negativamente la salud cardiovascular (EFSA, 2011) por lo que recomienda el consumo mínimo de 5 mg/día de HT.

A pesar de las muchas propiedades descritas *in vitro* por el HT son pocos los estudios que analizan los efectos *in vivo* de este compuesto, estas propiedades quedan recogidas en la **figura 6.**

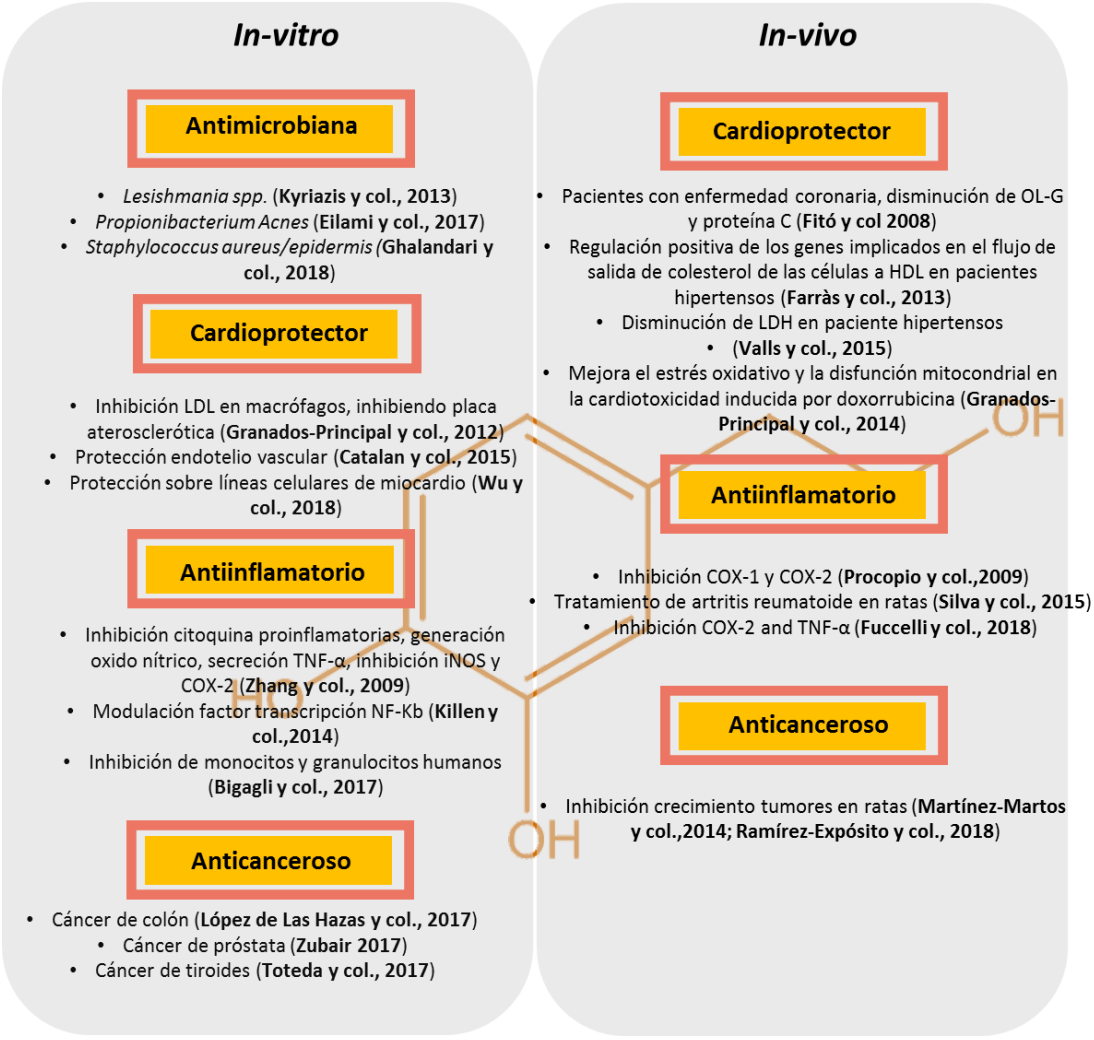

Figura 6. Resumen de las propiedades *in vitro* e *in vivo* del hidroxitirosol.

2.3.3 3,4-dihidroxifenilglicol (DHFG)

El tratamiento del subproducto de la extracción del aceite de oliva conduce a la recuperación y concentración de otros compuestos interesantes entre ellos el 3,4-dihidroxifenilglicol (DHFG), el cual es un fenol simple presente en la aceituna. Este compuesto ha sido aislado y purificado de los subproductos de aceite de oliva (**Rodríguez-Gutiérrez y col., 2009**) utilizando un procedimiento altamente adaptado y patentado (**Rodríguez-Gutiérrez y col., 2011**), del cual se obtiene el compuesto con una pureza

superior al 90% en peso seco. El DHFG tiene la misma estructura orto-difenólica que la HT, pero con un grupo hidroxilo adicional en la posición β.

A pesar de los muchos estudios que muestran la amplia variedad de actividades biológicas que tienen el hidroxitirosol, sin embargo, hasta el momento no se han realizado estudios sobre las posibles propiedades bioactivas y funcionales del DHFG, debido a su reciente disponibilidad (la presencia de un grupo hidroxilo adicional en su estructura respecto al hidroxitirosol le podría conferir mejores propiedades biológicas, o mejorar las que éste último ya posee. En nuestro grupo de investigación se han llevado a cabo los ensayos de la capacidad antioxidante del DHFG mediante cuatro ensayos in-*vitro*: capacidad anti-radical, poder reductor e inhibición de la oxidación primaria, secundaria en sistemas lipídicos (**Rodríguez-Gutiérrez y col., 2007b**). Se ha demostrado por primera vez que el DHFG tiene, en fase acuosa, una capacidad antioxidante 2-3 veces superior al hidroxitirosol y la vitamina C. Tiene también más capacidad antirradical y poder reductor que el hidroxitirosol y las vitaminas C y E. Se ha observado también que, en fase lipídica, tiene una capacidad similar a la vitamina E en la inhibición de la oxidación, a pesar de su alta polaridad. **Rodríguez-Guitiérrez y col**. (2012) realizan un estudio de los índices y mecanismos de estrés oxidativo y metabólico en ratas. Para ello utilizan ratas deficientes en vitamina E y posteriormente se suplementa esta dieta con α-tocoferol, un extracto fenólico, HT y DHFG. La biodisponibilidad de los compuestos fenólicos queda demostrada mediante su detección en sangre y tejidos. Estudios proteómicos realizados en este trabajo relevan que el α-tocoferol y los compuestos fenólicos regulan la proteína hepática mitocondrial aldehído dehidrogenasa (ALDH2) que representa un mecanismo antioxidante. Por tanto, se demuestra que los compuestos fenólicos de la aceituna o del aceite de oliva tienen impacto beneficioso sobre la salud cardiovascular. Estudios de inhibición de la agregación plaquetaria demostraron un efecto sinérgico entre HT y DHFG (**Rodríguez-Gutiérrez y col., 2012**). Así mismo otro estudio realizado con ratas deficiente en vitamina E ha demostrado el efecto sinérgico del HT y DHFG sobre la inhibición de la peroxidación de los microsomas hepáticos (**Rubio-Senent y col., 2015c**).

El DHFG podría tener un notable interés en el campo de la nutrición y la farmacología porque presenta un alto poder antioxidante y es además el principal metabolito producido por la desaminación del neurotransmisor humano noradrenalina (**Venneri y Del Rio, 2004**). Por tanto, DHFG es un compuesto susceptible de ser incluido en una dieta saludable para ejercer una acción protectora frente al estrés oxidativo mediante el bloqueo de los radicales libres y/o la interrupción de la cadena de peroxidación lipídica. El enriquecimiento de productos alimentarios con DHFG puede emplearse como una estrategia para desarrollar alimentos funcionales capaces de conferir beneficios sobre funciones del organismo, en la conservación de alimentos y en aplicaciones cosméticas como agente antienvejecimiento y antioxidante. Por lo tanto, puede ayudar al emergente y prometedor papel de los antioxidantes como herramienta terapéutica en la prevención y tratamiento de enfermedades neurodegenerativas.

2.3.4 Recuperación de compuestos fenólicos.

Durante los últimos años el interés en la recuperación de los compuestos fenólicos, en concentraciones hasta 100 veces más altas en los residuos del aceite de oliva que en el propio aceite (**Lesage-Meessen y col., 2001**), ha aumentado enormemente. La recuperación de estos compuestos a partir del alperujo, como parte de su aprovechamiento, puede cambiar la visión negativa que se tiene de este subproducto y reconocerlo como una fuente barata y una valiosa materia prima para la producción de compuestos bioactivos y antioxidantes naturales con un amplio espectro de actividades biológicas, particularmente para las industrias alimentarias, cosméticas y farmacéuticas. Ciertamente, su recuperación, aparte de por su valor económico intrínseco, podría también ser beneficiosa por la consiguiente reducción del contenido fenólico en vista de futuros tratamientos y/o aplicaciones (**Allouche y col., 2004**), dados los efectos fitotóxicos y antimicrobianos que poseen los compuestos fenólicos. Por lo tanto, la extracción de estos compuestos fenólicos como productos de

alto valor añadido podría ser considerada como una interesante alternativa para hacer provechosos los residuos de las almazaras.

Los principales sistemas propuestos para recuperar fenoles a partir de muestras de origen vegetal y aguas residuales de procesos industriales se encuentran recogidos en la **figura 7**.

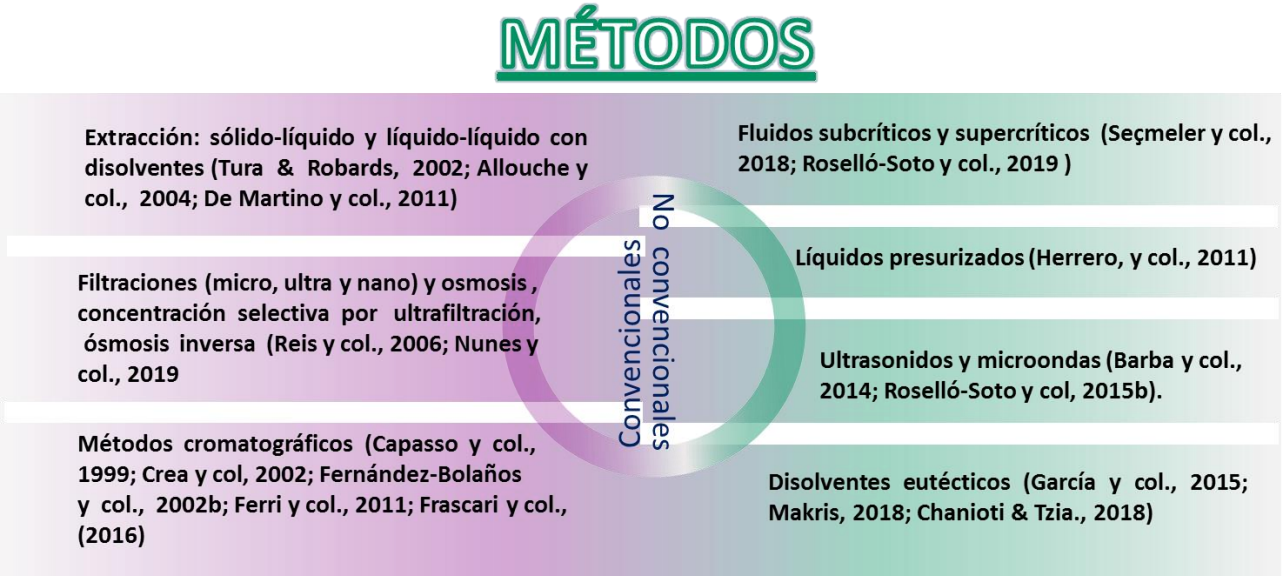

Figura 7. Esquema resumen métodos convencionales y no convencionales de extracción de compuestos fenólicos de origen vegetal.

Uno de los diferentes métodos de extracción mencionados, los métodos convencionales presentan en muchos casos el inconveniente del consumo de grandes cantidades de disolventes orgánicos.

Uno de los procedimientos descritos y patentado para la purificación de HT de forma simple, práctica y económica, que permite obtener HT a nivel industrial con alto grado de pureza (99,6%) y evitando el uso de disolventes orgánicos, es el método desarrollado en el grupo de Fitoquímicos, biactividad y desarrollo de procesos del Instituto de la Grasa (C.S.I.C.) (**Fernández-Bolaños y col., 2002b**). A partir de la fracción líquida obtenida tras el tratamiento térmico con vapor nuestro grupo ha aislado y purificado de forma sencilla, dos antioxidantes fenólicos muy activos presentes en la

aceituna, el HT y el DHFG. Los sistemas de purificación de estos dos antioxidantes naturales se han patentado (**Rodríguez-Gutiérrez y col., 2011; Fernández-Bolaños y col., 2013**) y, en el caso del HT se ha llevado a la industria y hoy se comercializa bajo el nombre de Olivefen®. La empresa SVM (Subproductos Vegetales del Mediterráneo S.L.), de base tecnológica (EBT), surge de nuestro grupo de investigación, con el objetivo principal de obtención y purificación del hidroxitirosol.

2.4 Polisacáridos

Composición y estructura de la pared celular vegetal.

La pared celular es un componente principal de la célula vegetal que le confiere protección, tanto de naturaleza física, como química y mecánica, y es esencial para muchos procesos del crecimiento vegetal, desarrollo, mantenimiento y reproducción. Es una estructura semirrígida que se encuentra rodeando a la membrana plasmática, y constituye la capa más externa de la célula. Está compuesta fundamentalmente de polisacáridos y pequeñas cantidades de glicoproteínas y compuestos fenólicos, contrastando con la membrana plasmática, de menor espesor, en la que fosfolípidos y proteínas son los componentes principales.

La pared celular vegetal se puede dividir en tres zonas: la lámina media, la pared celular primaria y la secundaria, que son el resultado de la evolución de una serie de etapas de diferenciación celular (**Fig. 8**)

- La lamina media es la más externa, común a dos células continuas, de la que constituye una especie de tabique. Es particularmente abundante en polisacáridos pécticos (pectinas).
- Tras la formación de la lámina media, se depositan a ambos lados de la misma carbohidratos, algunas glicoproteínas y compuestos fenólicos, originando la pared primaria, de estructura poco compleja, poco ordenada y dinámica. Esta capa se encuentra constituida principalmente por fibras de celulosa embebidas en una mezcla amorfa de polisacáridos y proteínas. La pared celular primaria es propia de células en crecimiento y forma la base estructural del esqueleto de la planta.

- Por último, cuando cesa el crecimiento de la célula, la pared puede seguir creciendo en grosor, dando lugar a la pared secundaria, únicamente presente en células diferenciadas con funciones específicas (traqueidas, vasos, etc…) (**Wilson, 1993**). A su vez está formada por tres capas que se distinguen estructuralmente por la distinta orientación de las microfibrillas de celulosa (S1, S2 y S3) (**Harris, 1990**). Generalmente, la aparición de la pared secundaria va acompañada de cambios en la composición química de la lámina media y de la pared primaria, tales como la lignificación, en esta fase la concentración de lignina es superior en estas dos capas (**Jiménez, 1993; Heredia y col., 1993; Heredia y col., 1995**). Se considera una pared suplementaria que realiza funciones mecánicas (**Monties, 1980**).

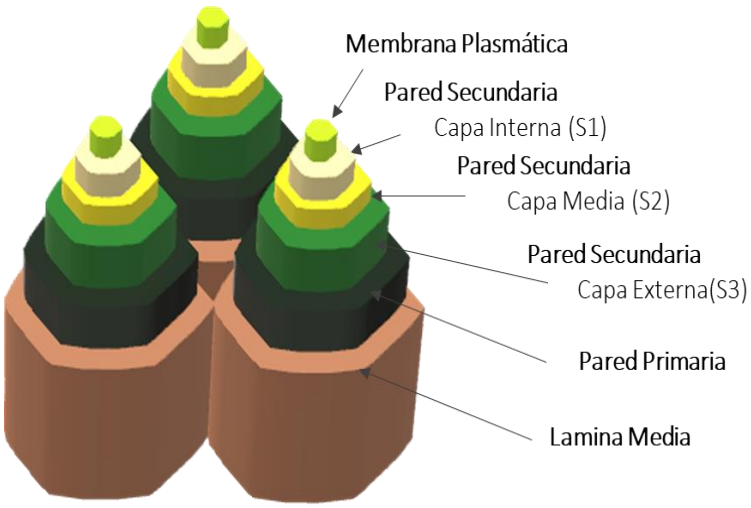

Figura 8. Estructura de la pared celular vegetal

<u>Componentes fibrosos de la pared celular.</u>

Las paredes primaria y secundaria presentan dos fases, una microfibrilar y otra amorfa (matriz), que no se da en la lámina media. La fase microfibrilar posee un alto grado de cristalinidad y tiene una composición química homogénea constituida principalmente por celulosa. Por lo contrario, la matriz es químicamente compleja.

a) Fase microfibrilar: celulosa

Esta fase está compuesta por microfibrillas largas y delgadas constituidas por moléculas de celulosa, las cuales se alinean paralelamente a su eje longitudinal. La celulosa es un polisacárido lineal de moléculas de glucosa (Glc) unidas por enlaces β

(1→4), de alto peso molecular (0.5-1 millón de Daltons), con un grado de polimerización superior a 15.000 en paredes secundarias, e inferior en paredes primarias. Presenta una estructura estable y altamente resistente debido a los puentes de hidrógeno intra- e intermoleculares. La configuración más estable es en forma de silla con los hidroxilos en posición ecuatorial.

b) Fase amorfa o matriz.

Esta fase se caracteriza por estar compuesta por una gran variedad de polisacáridos, junto a proteínas y a compuestos fenólicos. Todos estos compuestos están unidos entre sí y a las microfibrillas por fuerzas de distintos orígenes, y varían su composición según las diferentes partes de la pared celular, tipos de células, y estados del ciclo celular (**Brett & Waldron, 1990**).

Los componentes de la matriz se detallan a continuación.

b.1) Polisacáridos pécticos. Son componentes de la pared celular primaria se caracterizan porque el principal componente es el ácido D-galacturónico. En base a su estructura constituyen un grupo de polímeros asociados a residuos de ácido D-galacturónico, unidos por enlaces α (1→4). Además de ácido D-galacturónico, poseen importantes cantidades de ramnosa, arabinosa y galactosa. Generalmente la ramnosa forma parte de la cadena principal, mientras que la arabinosa y la galactosa se encuentran en las cadenas laterales (**Aspinall, 1983**) (**Fig. 12**). Constituyen una mezcla compleja de polisacáridos helicoidales. Los de alto grado de esterificación, tienen poca capacidad de interacción con otros componentes de la pared celular y por ello pueden extraerse con agua caliente. Los de medio o bajo grado de esterificación están estabilizados por enlaces iónicos con calcio, formando geles con estructura de "caja de huevos" y pueden solubilizarse en agentes quelantes (**Fig. 13**). Otros pueden estar enlazados por enlaces covalentes a hemicelulosas, celulosa o incluso proteínas y se separan sólo por tratamiento con álcali o con ácido diluido (**Van Buren, 1979**).

b.2.) Hemicelulosas. Son polisacáridos no celulósicos, que se pueden extraer de la pared celular con soluciones acuosas de álcalis diluidas, después de la eliminación de la lignina. A diferencia de la celulosa están compuestas de diferentes azúcares formando

cadenas más cortas y con ramificaciones. Los azúcares que la forman se pueden dividir en diferentes grupos, como las pentosas (xilosa y arabinosa), hexosas (glucosa, manosa y galactosa), ácidos hexurónicos (ácido glucurónico, metilglucurónico y galacturónico) y desoxihexosas (ramnosa y mucosa). La cadena principal de una hemicelulosa puede consistir en una sola unidad que se repite (homopolímero), o en dos o más unidades (heteropolímero). La mayoría de las hemicelulosas son heteropolisacáridos complejos que contienen entre dos y cuatro tipos de azúcares (**Southgate, 1990**). Se caracterizan porque se hidrolizan fácilmente en medio ácido, obteniéndose como principales constituyentes monoméricos D-glucosa, D-manosa, Dgalactosa, D-xilosa y L-arabinosa y también pequeñas cantidades de D-glucurónico, 4-O-metil-D-glucurónico y D-galacturónico (**Sjöström, 1982**). Muchas hemicelulosas se encuentran unidas a las microfibrillas de celulosa por puentes de hidrógeno y también hay evidencias que sugieren uniones covalentes entre hemicelulosas y polisacáridos pécticos y entre éstas y lignina.

b.3.) Lignina y otros componentes fenólicos. La lignina es un polímero estructural que aparece en ciertos tipos de células diferenciadas una vez que la elongación celular ha finalizado. Está constituido por tres redes tridimensionales de unidades de fenilpropano unidas por enlaces C-O-C (éter) y C-C, siendo los primeros mayoritarios (**Sjöström, 1982**). Los precursores de la lignina son los alcoholes aromáticos, cumarílico, coniferílico y sinapílico, que se originan a partir de la D-glucosa a través de reacciones enzimáticas complejas, y se unen para formar el polímero final.

La polimerización de la lignina comienza en la lámina media, extendiéndose a la pared primaria y posteriormente a la secundaria, siempre que exista espacio disponible, de modo que tiende a rellenar todo el espacio libre que no está ocupado por macromoléculas, desplazando para ello al agua. El resultado es un entramado hidrofóbico muy fuerte que rodea a los otros componentes de la pared y los mantiene unidos.

La lignina determina las características de la fibra vegetal. Su presencia en la pared es la responsable de la dificultad de degradar la fibra, ya que forma una pantalla

que dificulta la accesibilidad de agentes químicos y/o enzimáticos a los otros componentes de la fibra, existiendo una correlación entre el contenido de lignina en la pared celular y la digestibilidad de la misma (**Jung & Deetz, 1993**). Los componentes más digeribles de la pared son las sustancias pécticas, mientras que las hemicelulosas y celulosas que están protegidas por la barrera de lignina y posiblemente unidas a ellas no son digeribles (**Wilson y col., 1989**).

Además de la lignina en la pared celular pueden encontrarse otros fenoles, principalmente el ácido ferúlico, que se halla esterificado con la arabinosa y la galactosa de los polisacáridos pécticos y tiene un papel importante en las uniones transversales de las mismos. También se encuentra el ácido cumárico que principalmente está esterificado y su concentración aumenta con la maduración (**Liyama y col., 1990**).

b.4.) Proteínas. Se clasifican según sean estructurales o enzimas. Las proteínas estructurales están glicosiladas y enriquecidas en el aminoácido hidroxiprolina, conteniendo además alanina, serina y treonina (**Showalter, 1993**). Por otro lado, las enzimas se encuentran en menor proporción que las estructurales, en este grupo se incluyen peroxidasas e hidrolasas.

Todos los polímeros descritos están enlazados entre sí por diferentes uniones, siendo éstas las que determinan una estructura que funciona como unidad. El conjunto es mantenido por distintos tipos de enlaces intermoleculares y de interacciones fisicoquímicas que confieren a la célula gran resistencia mecánica y hacen que la pared celular sea resistente a los agentes químicos y fisicoquímicos (**Hatfield, 1993**). En el seno de este conjunto la celulosa, polímero en parte cristalino y orientado en forma de microfibrillas, constituyen una trama que sirve de sostén a los otros compuestos poliméricos (**Fig. 9**).

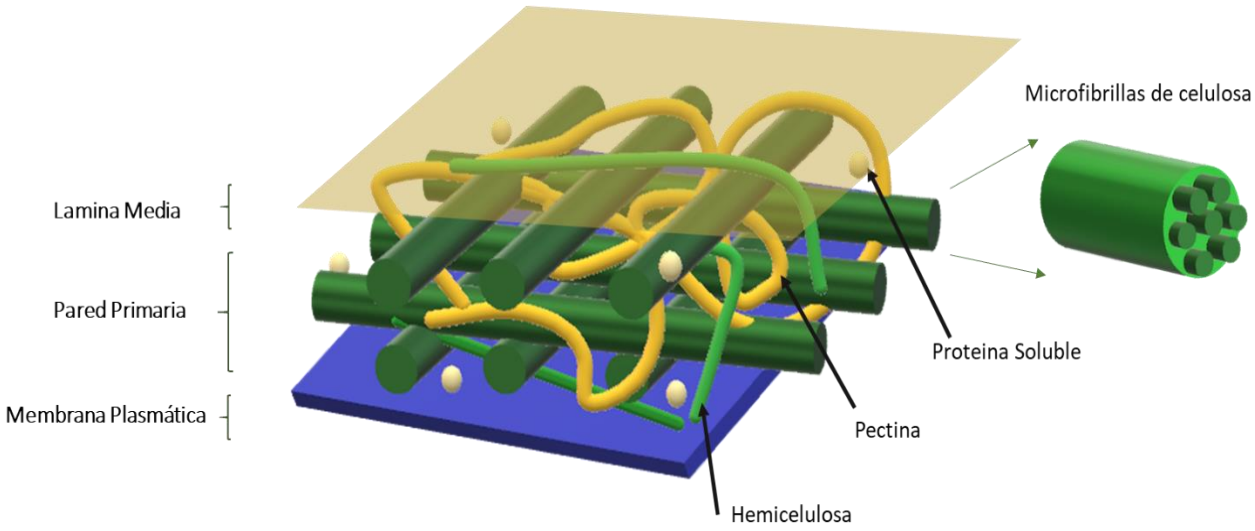

Figura 9. Estructura de la pared celular, disposición de los diferentes componentes.

2.4.1 Pared celular de la aceituna

La pared celular vegetal consiste por tanto en una mezcla compleja de polisacáridos, los cuales se suelen denominar como fibra alimentaria. La fibra alimentaria del fruto de la oliva incluye pectinas, hemicelulosas (enriquecidas en xilanos, xiloglucanos, glucurunoxilanos y mananas), celulosa y lignina (**Coimbra y col., 1994**).

La pared celular de la pulpa del olivo contiene sobre un tercio de polisacáridos pécticos con un elevado grado de esterificación (>80%). Las pectinas se encuentran formadas por una fracción neutra compuesta por arabinanos principalmente, mientras que la fracción ácida se encuentra formada por homogalacturanos y ramnogalacturanos (**Jiménez y col., 1994**). El fruto de la oliva puede estar parcialmente lignificado, principalmente en las zonas cercanas al hueso, en esta zona el contenido de xilanos es elevado mientras que la concentración de pectinas desciende. Igualmente, la zona del endocarpio se encuentra altamente lignificada, presentando elevadas concentraciones de celulosa y hemicelulosas (**Coimbra y col., 1995**). Las pectinas están formadas por una cadena principal de ácido galacturónico enlazados por uniones α (1→4) que presentan de forma intercalada residuos de ramnosa con enlaces α (1→2) que sirven de anclaje para cadenas laterales por el C-4, de residuos de arabinosas enlazados por uniones α (1→2).

2.4.2 Fibra alimentaria y función.

Los polisacáridos de las paredes celulares vegetales (celulosa, hemicelulosas y pectinas) son junto con la lignina los principales constituyentes de la denominada fibra alimentaría. Se caracterizan porque no son digeridos por las enzimas humanas, pero sí son parcialmente fermentados por las bacterias del colon pudiendo actuar como prebióticos (**Mitchell & Tiihonen, 2003**). Hoy en día, las dietas de los consumidores están cambiando hacia una dieta más rica en frutas, verduras y leguminosas, los cuales presentan un alto nivel nutricional (**van Dooren y col., 2017**) siendo, además, las principales fuentes de fibra alimentaria.

La fibra alimentaria es el conjunto de fibra insoluble y soluble, estando la primera compuesta principalmente por componentes de la pared celular como celulosa y lignina, y la segunda compuesta por polisacáridos no celulósicos, tales como las pectinas

La fibra insoluble parece acelerar el paso de los alimentos a través del estómago y los intestinos y les agrega volumen y consistencia a las heces, siendo escasamente fermentada. Mientras que la fibra soluble retiene el agua, forma soluciones viscosas, retarda la digestión y la absorción de nutrientes desde el estómago y el intestino. Esta fibra es fermentada mayormente en el colón o intestino grueso por la flora intestinal.

La Organización Mundial de la Salud (OMS) fija un consumo mínimo de fibra de 30 g/persona /día, de la cual el 30% debe ser fibra soluble. La necesidad de consumir unos niveles mínimos radica en que un déficit del consumo de fibra conlleva a la aparición de enfermedades crónicas y funcionales como el estreñimiento, enfermedad inflamatoria intestinal, apendicitis, síndrome del colon irritable y cáncer de colon (**Guarner, 2003; Blaut, 2002**). Estos efectos beneficiosos se deben, por un lado, a las propiedades prebióticas que presenta la fibra en la microbiota intestinal, (**Tao y col., 2019**) y por otro lado a las propiedades físico-químicas. La capacidad de captación de moléculas orgánicas, está directamente implicada en la disminución de glucosa en sangre y por ende control de la diabetes (**Rubio-Senent y col., 2015a**). La fibra rica en pectinas presenta la capacidad de secuestrar o incluso unirse químicamente a los ácidos biliares (**Rubio-Senent y col., 2015a).** Aumentar la velocidad de paso de los alimentos por el

tracto intestinal, permite que los agentes carcinogénicos permanezcan menos tiempo de contacto con el tracto, así como la captación de ellos (**Ferguson & Harris, 1996**). La capacidad de retención de lípidos y colesterol está directamente relacionada con la disminución de sufrir problemas cardiovasculares (**Carr y col., 1996**). Es por ello que existe una fuerte evidencia en la que una ingesta enriquecida en fibra (soluble e insoluble) de cereales, leguminosas, verduras y fruta, muy abundante en la dieta mediterránea, tiene un efecto beneficioso previniendo enfermedades cardiovasculares, el estreñimiento, la diabetes, la obesidad y el cáncer. (**Bazzano y col., 2003**; **Willem y col., 2010**). Es por tanto que las propiedades físico-químicas, así como las propiedades funcionales que presentan la fibra alimentaria dependerán en gran medida de la proporción que exista de fibra soluble y fibra insoluble (**Fig. 10**)

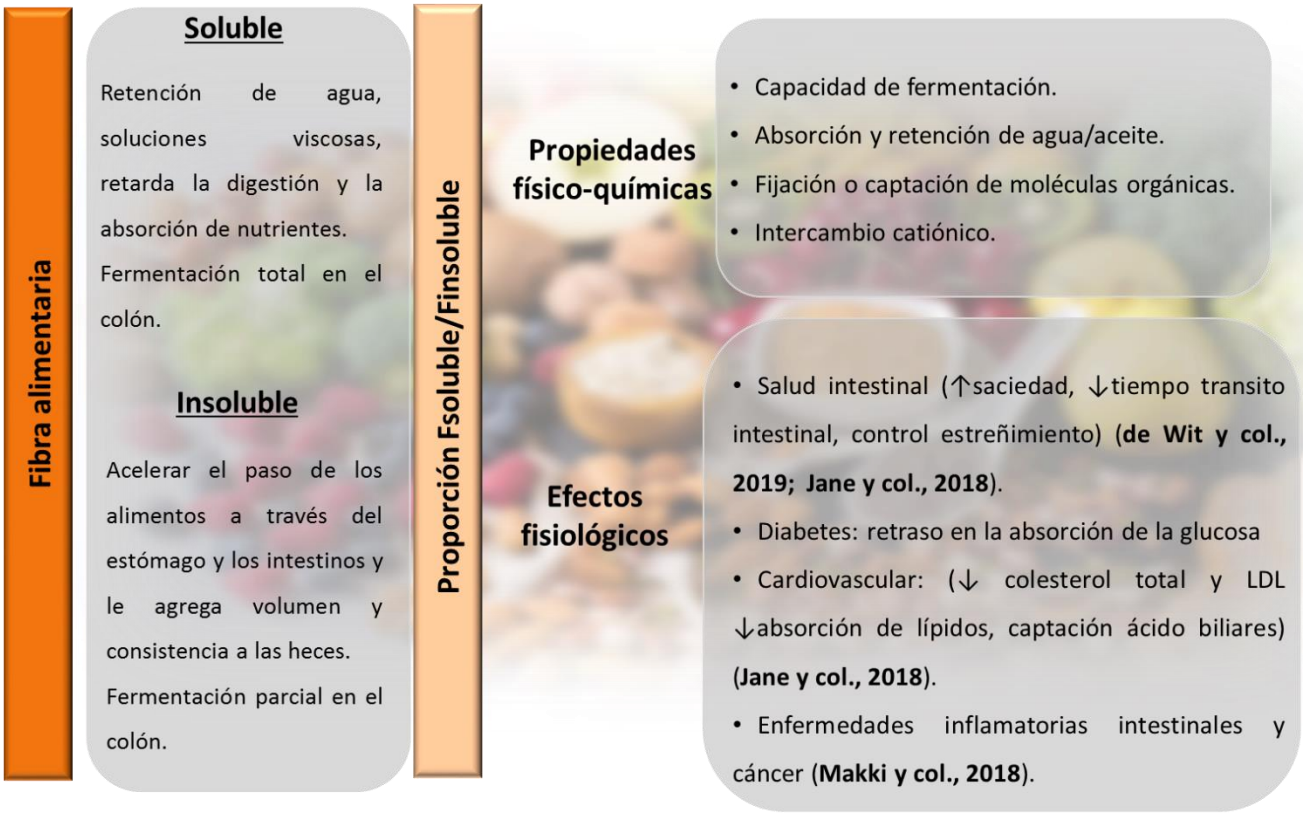

Figura 10. Esquema fibra alimentaria y propiedades

2.4.2.1 Fibra alimentaria antioxidante

Desde hace más de una década se ha comenzado a introducir un nuevo concepto dentro de la fibra alimentaria y es "fibra alimentaria antioxidante", la cual podría ser considerada como un potente ingrediente funcional (**Saura-Calixto,1998**).

La unión de estos dos componentes (fenol-fibra) podría mejorar la calidad de la fibra produciendo un importante efecto sinérgico. Vitaminas, polifenoles y carotenoides son los principales antioxidantes presentes en la dieta. Los polifenoles son un complejo grupo de sustancias con un amplio rango de pesos moleculares que se encuentran en las plantas bien en forma libre o enlazados a los constituyentes de la pared celular (fibra alimentaria). Se ha comprobado que la presencia de polifenoles y carotenoides asociados a la fibra alimentaria es una característica común a todos los alimentos vegetales (**Saura-Calixto & Díaz-Rubio, 2007**). De hecho, los polifenoles se unen forma rápida y espontánea a los polisacáridos de la pared celular de los alimentos ricos en fibra alimentaria cuando éstos son liberados por la ruptura de frutas y verduras durante la comida (molienda, masticación) o el propio procesamiento de los alimentos (ebullición, autoclave o liofilización) (**Liu y col., 2017**). Por lo tanto, la bioaccesibilidad de los polifenoles se vería afectada por estas interacciones (**Liu y col., 2017, Padayachee y col., 2017**). De hecho, se pueden diferenciar dos tipos de polifenoles en los alimentos, los polifenoles fácilmente extraíbles (biodisponibles en el tracto gastrointestinal humano) y los polifenoles no extraíbles o asociados a la fibra alimentaria (**Pérez-Jiménez & Saura-Calixto, 2018**). Estos fenoles formarían un complejo fenol-polisacárido, en el cual la interacción podría ser de tipo hidrofóbicas, enlaces covalentes y/o mediante enlaces de hidrógeno entre los grupos hidroxilo de los fenoles y los átomos de oxígenos de los polisacáridos (**Fig.11**) En general, los polifenoles interaccionan con el grupo carboxilo del ácido urónico (hemicelulosa y pectina) y con el grupo hidroxilo presente en la celulosa (**Metzler & Mosenthin, 2008**).

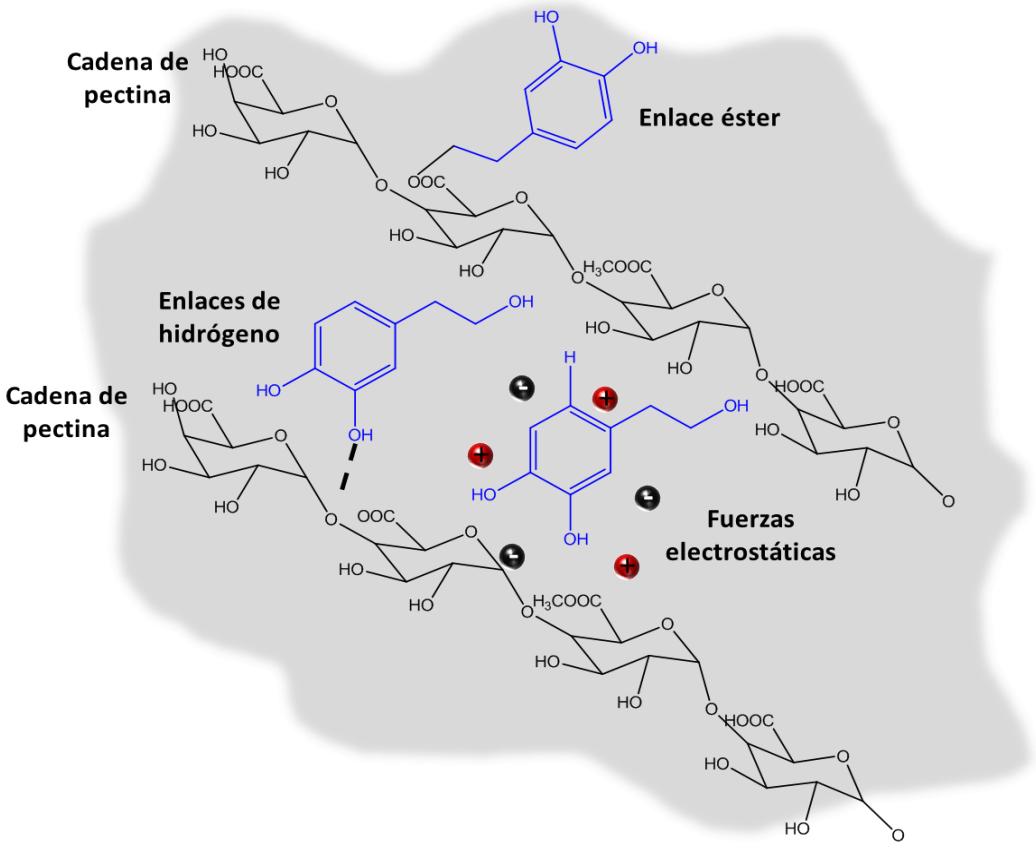

Figura 11. Diferentes interacciones entre compuestos fenólicos (HT) y cadenas de pectinas.

Estos compuestos antioxidantes unidos a la fibra no se disuelven ni absorben en el intestino delgado y llegan inalterados al intestino grueso donde se degradan, actuando la fibra como transportadora de antioxidantes en el tracto intestinal. Además, los compuestos fenólicos asociados con fibra dietética soluble e insoluble pueden eliminar los radicales libres, protegiendo las células contra el daño oxidativo, promoviendo la salud intestinal (**Pérez-Jiménez & Saura-Calixto., 2015**). De hecho, son muchos los estudios que actualmente han asociado la fibra alimentaria antioxidante con la prevención de cáncer de colon (**Arranz y col., 2010; Saura-Calixto, 2011; López-Oliva y col., 2010**). Por tanto, la fibra alimentaria asociada a compuesto fenólicos es un prometedor material tanto para la industria alimentaria como para el campo de la nutrición ya que combina las propiedades de dos compuestos en un único material.

2.4.3 Pectinas

Las pectinas representan una familia de complejos heteropolisacáridos aniónicos presentes en las paredes celulares de vegetales, frutas y otras plantas, considerándose fibra soluble. Son una mezcla de polímeros ácidos y neutros muy ramificados. El miembro más conocido y predominante es el homogalacturonano (HG), conocido como la "región suave" de la pectina, compuesto por una cadena lineal de moléculas de ácido α-(1,4)-D-galacturónico cuyos grupos carboxílicos se encuentran parcialmente metoxilados (AGal). Otro miembro bien caracterizado de las pectinas, conocido como "región densa" o región de Ramnogalacturano I (RGI), consiste en una cadena principal compuesta por AGal enlazado con restos de ramnosa (Ram), es decir [(1-4)- α -D-AGal-(1,2)-a-L-Ram-]n (**Fig.12**). La RGI está muy ramificada con azúcares neutros (principalmente α –L-arabinosa y β-D-galactosa). Los restos de Rha son el anclaje a las cadenas laterales de arabinados, galactanos y arabinogalactanos. Los extractos de pectinas también suelen contener Xilogalacturanos (XG), y Ramnogalacturanos II (RGII), una estructura ramificada y compleja que se da con menos frecuencia que la RGI.

Algunos de los grupos carboxílicos de los residuos galacturónicos pueden estar esterificados (generalmente metilados). El grado de esterificación de una pectina variará entre 0 y 90%. Si el 50% o más de los grupos carboxílicos están esterificados, entonces se aludirá a la pectina resultante como una "pectina de elevado grado de esterificación" (pectina HE). Si menos del 50% de los grupos carboxilos están esterificados, entonces se aludirá a la pectina resultante como a una "pectina de bajo grado de esterificación" ("pectina LE").

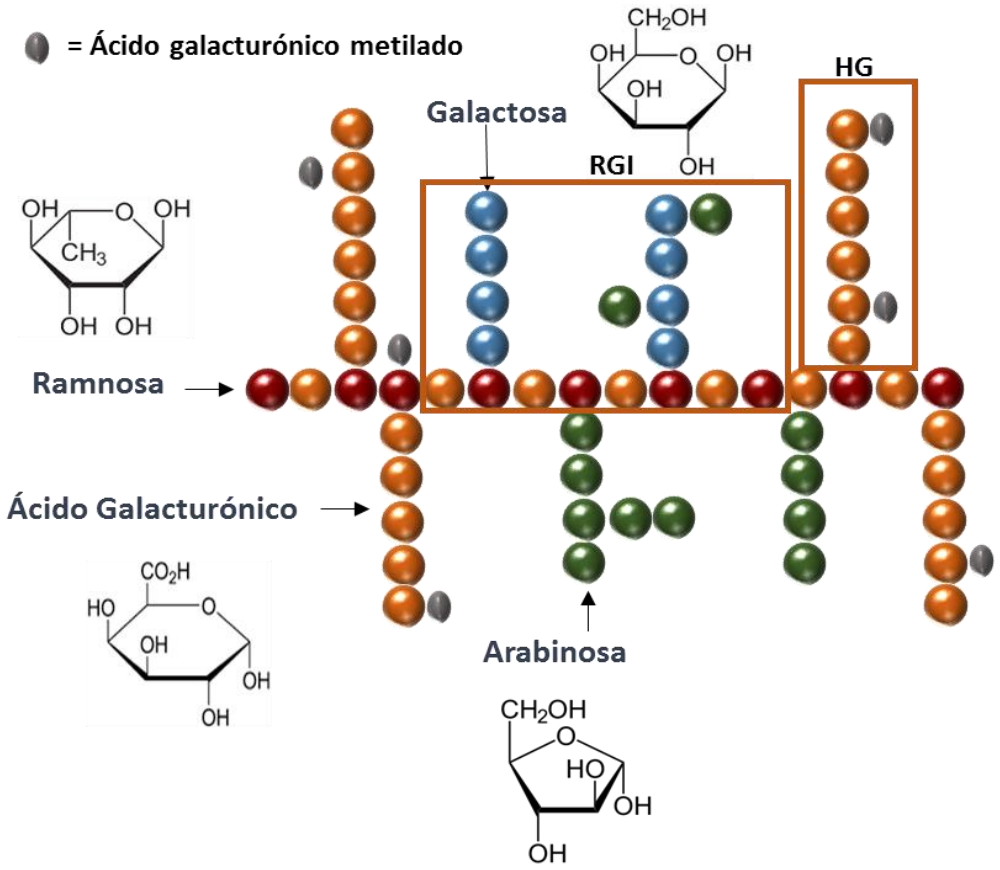

Figura 12. Estructura de la pectina

El grado de esterificación va determinar sus propiedades físicas y/o químicas. Por ejemplo, la gelificación de la pectina depende de su naturaleza química, en particular de su grado de esterificación. Además, sin embargo, la gelificación péctica depende también del contenido de sólidos solubles, del pH y de la concentración de iones cálcicos. En el caso de las pectinas de bajo metoxilo, la gelificación ocurre mediante uniones de iones calcio (Ca^{2+}), que forman puentes entre las cargas negativas de las cadenas de pectinas, donde lugar a una estructura semejante a una "caja de huevos" (**Fig. 13**). Mientras que las pectinas de alto metoxilo la felificación ocurre mediante interacciones hidrofóbicas de los grupos metoxilo o mediante puentes de hidrógeno, incluidos los de los grupos ácidos no ionizados. Esto se logra gracias a la existencia de un material hidrófilo como puede ser el azúcar, la cual retira el agua y permite las uniones.

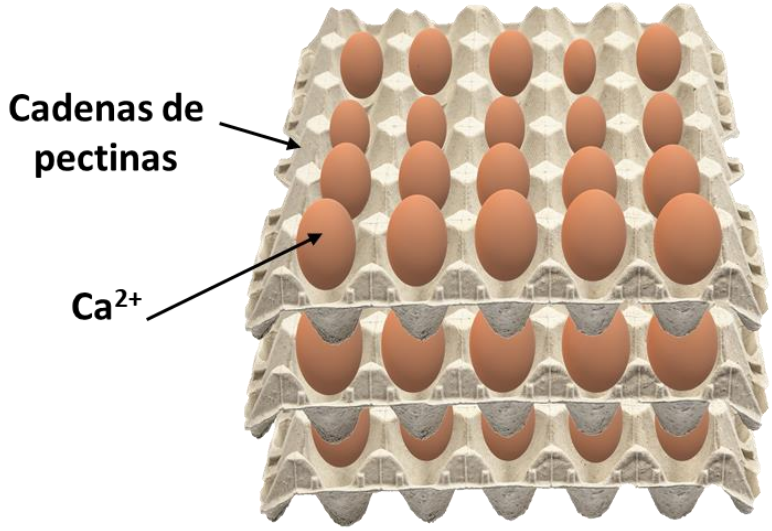

Figura 13. Modelo caja de huevos

Las propiedades fisicoquímicas que estas presentan han permitido su uso como agentes gelificantes, estabilizantes, e emulsionantes. Sin embargo, las pectinas presentan un amplio abanico de propiedades biológicas debido a su fermentabilidad. Hoy en día el interés radica en los oligosacáridos pécticos como una nueva clase de prebióticos capaces de ejercer efectos beneficiosos para la salud, propiedades anticancerígenas *in vitro*, protección cardiovascular, así como propiedades antibacterianas, antiinflamatorias y antioxidantes. (**Olano-Martin y col., 2002; Maxwell y col., 2015; Samuelsson y col., 2016**)

Las pectinas comerciales actualmente se obtienen a partir de limón o manzana, aunque en los últimos años se ha visto incrementado el interés por obtenerlas a partir de otras fuentes vegetales, sobre todo a partir de subproductos de la industria alimentaria. Existen numerosos procesos patentados para obtener pectinas, y en cada uno de ellos se obtienen productos de diferente calidad en función del método de obtención empleado. Comercialmente, la pectina se extrae principalmente a partir de los subproductos obtenidos de manzana y cítricos como las naranjas y los limones (**May, 1990**). Los métodos convencionales para la extracción de pectina se basan en el uso de ácidos como

agente extractante (HCl, H_2SO_4, HNO_3) a temperaturas comprendidas entre 60 y 100°C (**Chan y col., 2013; de Oliveira y col., 2015**; **Hosseini y col., 2016**). También se realiza extracciones con ácido cítrico, con el cual se obtienen rendimientos similares o incluso mayores, debido principalmente al efecto quelante (**Maneerat y col., 2017**). Así mismo frente a los métodos convencionales se han ido desarrollado diferentes métodos de extracciones de pectinas, como pueden ser; métodos enzimáticos (**Wilkiera y col., 2016**), extracciones asistidas por microondas (**Seixas y col., 2014**; **Lefsih y col., 2017**), el uso de campos eléctricos (**Yang y col., 2016**) y extracciones asistida por ultrasonidos (**Wang y col., 2016; de Oliveira y col., 2016**)

2.5 Recubrimientos comestibles

La razón primordial del uso de recubrimientos comestibles o películas comestibles es que alarga la vida útil de alimentos perecederos, en este sentido se deben aplicar técnicas de conservación que combinadas o no puedan mantener o mejorar las características sensoriales y nutricionales asegurando además su estabilidad microbiológica (**Rojas-Grau y col., 2009**). Los recubrimientos comestibles o películas comestibles han encontrado grandes aplicaciones en el campo de los alimentos entre lo que se encuentran: mejora de la vida útil de los productos frescos, reducción de la absorción de aceite durante el proceso de fritura, así como reducir la pérdida de color y aroma de los alimentos durante el almacenamiento (**Khazaei y col., 2016**). La diferencia entre un recubrimiento comestible y una película comestible es que el primero se define como una capa delgada de material formado sobre el alimento, mientras que el segundo es una capa delgada preformada que se coloca sobre el alimento (**McHugh, 2000**).

Las películas y recubrimientos comestibles deben cubrir los siguientes requerimientos para poder ser utilizados en alimentos: Buenas cualidades sensoriales, eficiencia mecánica y gran capacidad de barrera (CO_2, O_2, H_2O), suficiente estabilidad bioquímica, física y microbiológica, libre de tóxicos y seguros para la salud, tecnología simple de fabricación y aplicación, no contaminante y bajo coste como materia prima y proceso. (**McHugh & Kronchta, 1994b; Gutierrez y col., 2017**).

Los principales compuestos para la formulación de las películas o recubrimientos comestibles se encuentran recogidos en la **figura 14**. Los más usados son los polisacáridos, proteínas y lípidos, los cuales se pueden usar de forma aislada o combinada.

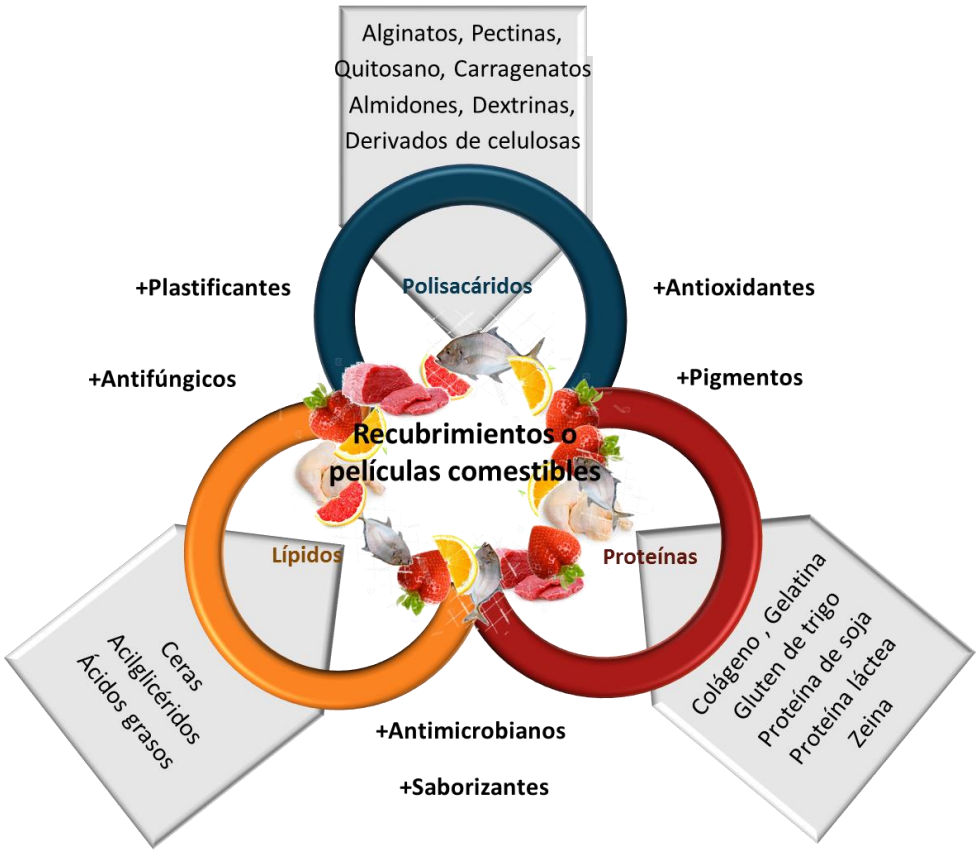

Figura 14. Esquema diferentes componentes de recubrimientos o películas comestibles.

Aunque los recubrimientos basados en polisacáridos son uno de los más utilizados debido a las propiedades de adhesión mecánica y a la flexibilidad de los mismos. Exhiben barreras selectivas al O_2 y el CO_2 (**Nísperos-Carriedo 1994; Nussinovith, 1997**) y una barrera pobre al vapor de agua (**Kester & Fennema, 1986, García y col., 1998**) debido a la naturaleza hidrofílica de estos. Los recubrimientos hechos de proteínas

exhiben mejores propiedades de barrera a los gases, pero tienen baja resistencia al vapor de agua debido a su naturaleza hidrófila (**Pérez-Gago & Krochta, 2002**). Los pares proteína-polisacárido, en particular, tienen un gran potencial para su uso en un gran número de complejos estructurales y coacervados con propiedades fisicoquímicas mejoradas, que si la película de proteína o polisacárido se emplearan de manera individual. (**Kester & Fenneman, 1986**). Mientras que los recubrimientos compuestos por lípidos, y debido a su polaridad, se emplean principalmente para evitar la pérdida de humedad.

Los recubrimientos o películas comestibles, pueden formularse en base a una proteína, carbohidrato o lípido. Individualmente, cada material puede formar películas o recubrimientos con propiedades específicas, pero el uso de combinaciones de materiales y la adición de plastificantes y surfactantes son generalmente uno de los enfoques para mejorar sus propiedades finales (**Campos, y col., 2010**). Por tanto, además del componente de naturaleza polimérica, el uso de aditivos, como los plastificantes, para mejorar la resistencia y flexibilidad de la película es generalmente necesario. Un plastificante se define como una sustancia no volátil, la cual es añadida a otro material y su función es atenuar las fuerzas intermoleculares entre las cadenas de polímeros adyacentes, lo que origina un decremento en la resistencia a la tensión y un incremento en la flexibilidad de las películas (**Gennadios & Weller, 1990; McHugh & Krochta, 1994a**). Algunos ejemplos de plastificantes de grado alimentario son; los polioles, tales como el glicerol, sorbitol, manitol, sacarosa, propilenglicol y polietilenglicol (**McHugh y Krochta, 1994a**). El sorbitol y el glicerol son plastificantes efectivos, debido a su potencialidad para reducir los puentes de hidrógeno internos, mientras incrementan el espaciado intermolecular (**Donhowe & Fennema, 1993**) lo que origina un descenso en la fragilidad, con el consiguiente aumento en flexibilidad (**McHugh & Krochta, 1994a**). Los plastificantes afectan la capacidad de atraer agua al sistema y generalmente aumenta la permeabilidad al oxígeno (**McHugh & Krochta, 1994a; Sothornvit & Krochta, 2000**).

A pesar de que los recubrimientos o películas comestibles aún no se consideran un sustituto comparado con las formas de envasado más tradicionales, presenta

numerosas ventajas (**Galus y col., 2015; Guimarães y col., 2018**), ya que, además, de las propiedades organolépticas, nutricionales y de conservación de los alimentos, pueden realzarse mediante la incorporación de agentes saborizantes, pigmentos, aditivos nutricionales agentes antimicrobianos, conservadores y/o agente antioxidantes a las películas comestibles que los envuelven mejorando las propiedades funcionales del propio alimento (**Baldwin y col., 1995; Gutiérrez, 2017**).

2.6 Encapsulados

La vía oral sigue siendo una de las vías más utilizadas para la administración de medicamentos (**Chien, 1992**). Actualmente muchos estudios se centran en el desarrollo de matrices que permitan la liberación controlada de fármacos en el tracto gástrico intestinal de ahí que la encapsulación de compuestos activos haya experimentado avances rápidos y significativos lo que han permitido su uso en las ramas más diversas de la industria, principalmente farmacéutica, cosmética y alimentaria (**Zanetti y col., 2018**). Generalmente estas formulaciones son sencillas, económicas y de fácil escalado a nivel industrial (**Li y col., 2005**).

El principio de encapsulación consiste simplemente en atrapar una sustancia activa para su posterior liberación a través de cambios en la fase gel en respuesta a estímulos externos (**Lakkis y col., 2007**). Diferentes mecanismos de activación se utilizan para liberar la sustancia activa, ya sea mediante cambio de pH, enzimas, desgaste mecánico y/o fuerzas osmóticas (**Maestrelly y col., 2007**) El tamaño de los encapsulados varían desde escala nanométrica (nanoencapsulación), micrómetrica (microencapsulación) o escala milimétrica (**Burgain y col., 2011**), existiendo un elevado número de técnicas de encapsulación (**Fig. 15**). Las principales ventajas que presenta la encapsulación es la posibilidad de: liberación controlada del compuesto encapsulado en una zona determinada, protección del material activo de, luz aire, humedad, modificación de las características originales, disimular sabores y olores, separación de componentes y estabilización (**Parra-Huertas, 2010**).

Figura 15. Esquema resumen del proceso de encapsulación y liberación.

La gelación iónica es una de las técnicas empleadas para la formación de perlas, las cuales se forman por polimerización iónica. El proceso consiste en la interacción de una solución polimérica acuosa con otra de iones de carga opuesta y de bajo peso molecular (ej: Ca^{+2}) (**Fig.16**) (**Burey y col., 2008**).

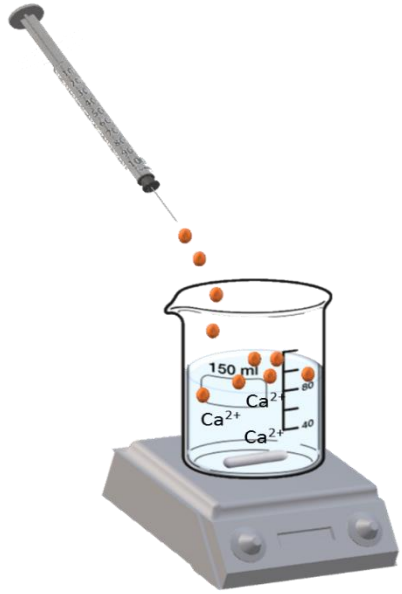

Figura 16. Proceso de elaboración encapsulados, método gelación iónica

Es un procedimiento simple y fácil, no requiere equipo especializado, altas temperaturas ni disolventes orgánicos por lo que puede considerarse de bajo coste (**Patil y col., 2010; Comunian, 2016**). El alginato, la pectina de baja metoxilación, la quitina, el quitosano son uno de los polímeros más ampliamente usados como agentes de recubrimiento, actuando de contraanión el Ca^{2+}. Se considera un buen sistema de encapsulación ya que no son tóxicos, son biocompatibles y presentan buenas propiedades mecánicas (**Chan y col., 2006; Lakkis y col., 2007; Patil y col., 2010**).

Las pectinas han sido ampliamente estudiadas como excipiente en diversas formas farmacéuticas para modular la liberación de moléculas activas. Esta permanece intacta en la parte alta del tracto gastrointestinal, y solo es degradada por la microflora del colon, por lo que muestran el gran potencial para modular la liberación específica de moléculas activas en dicha región. (**Das & Ng, 2010**). Además, se ha encontrado que inhibe la inflamación tanto local como sistémica y previene la inflamación intestinal (**Markov y col., 2011; Popov y col., 2013**).

2.7 Pectinas modificadas

Actualmente existe un interés en mantener un estilo de vida saludable para reducir la aparición o para evitar la progresión de enfermedades crónicas (**Morris y col., 2013**). Recientemente se ha empezado emplear el término “pectinas modificadas” (PMs) para referirse a pectinas de diferentes composición y características comunes. Son pectinas que han sido modificadas por tratamiento químicos con ácidos o álcali, por tratamiento térmico, radiación, ultrasonido y por tratamientos enzimáticos. Estos

tratamientos provocan cambios estructurales en la pectina, produciendo una despolimerización efectiva por hidrólisis y/o β-eliminación de las cadenas de homogalacturanos, dando lugar a la formación de fragmentos de bajo peso molecular lo que facilita su absorción en el tracto intestinal (**Raz y Pienta, 1998**) (**Fig. 17**).

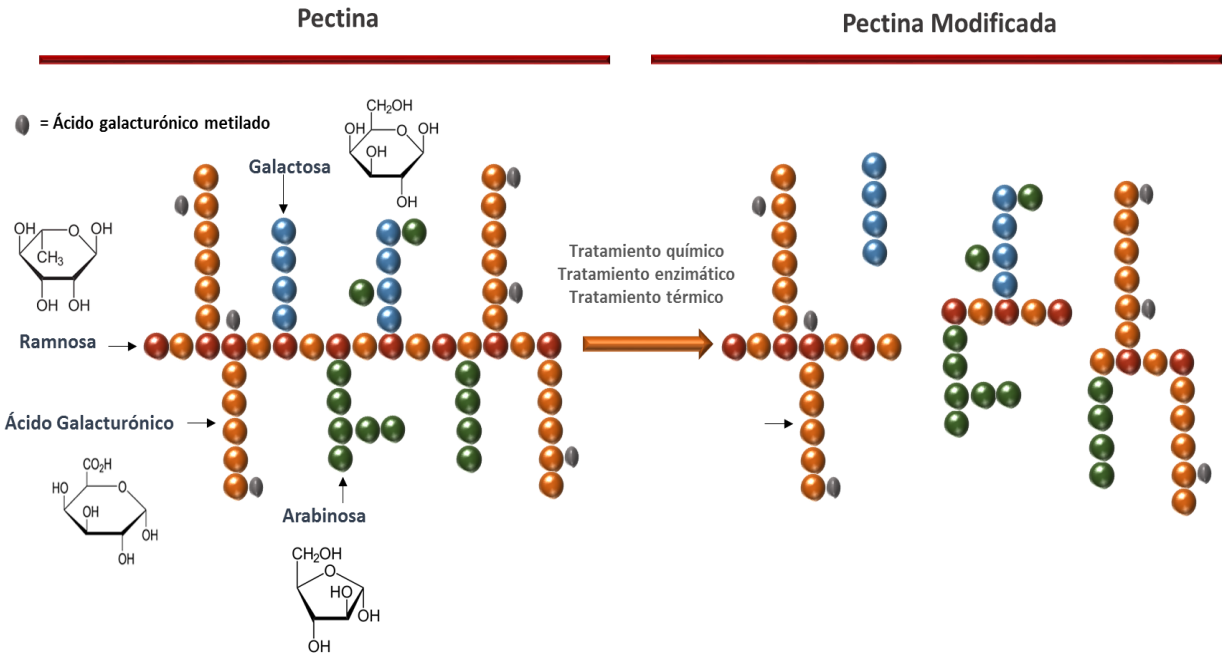

Figura 17. Obtención y esquema de una pectina modificada

Actualmente, la mayoría de las investigaciones realizadas con pectinas modificadas emplean pectinas obtenidas a partir de cítricos (**Jackson y col., 2007; Fang y col., 2018**) o manzana (**Li y col., 2012**). Las pectinas modificadas de limón obtenidas por cambio de pH mostraron inhibición del crecimiento tumoral, metástasis y la angiogénesis (**Glinsky & Raz., 2009; Grous y col., 2006**; **Fang y col., 2018**) Mientras que las pectinas modificadas de limón obtenidas mediante tratamientos térmicos mostraron acción apoptótica sobre líneas celulares de cáncer de próstata. (**Jackson y col., 2007**). Así como efectos positivos en el tratamiento y la prevención de enfermedades inflamatorias y relacionadas con la fibrosis (**Forsman y col., 2011; Okamura y col., 2011**).

Estos fragmentos pécticos de bajo peso molecular son capaces de competir con la galectina-3 (**Gao y col., 2013**). Esta proteína es miembro de la familia de las lectinas con acción β-galactosido, la cual presenta un sitio de reconocimiento de carbohidratos que específicamente reconoce a unidades de β-galactosido. Existen evidencias clínicas que muestran que la sobreexpresión de Gal-3 está asociada con la carcinogénesis y el potencial maligno de cánceres agresivos (tal como, vejiga, pulmón) (**Canesin y col., 2010; Vuong y col., 2019)**. Así se ha mostrado que la Gal-3 está implicada en el crecimiento, proliferación (**Honjo y col., 2001**), diferenciación y adhesión celular, angiogénesis (**Traini y col., 2014**), apoptosis (**Akahani y col., 1997**; **Nangia-Makker y col., 2007**), progresión del tumor y metástasis (**Radosavljevic y col., 2011**), a través principalmente de su unión a glicoproteínas (**Fig. 18**)

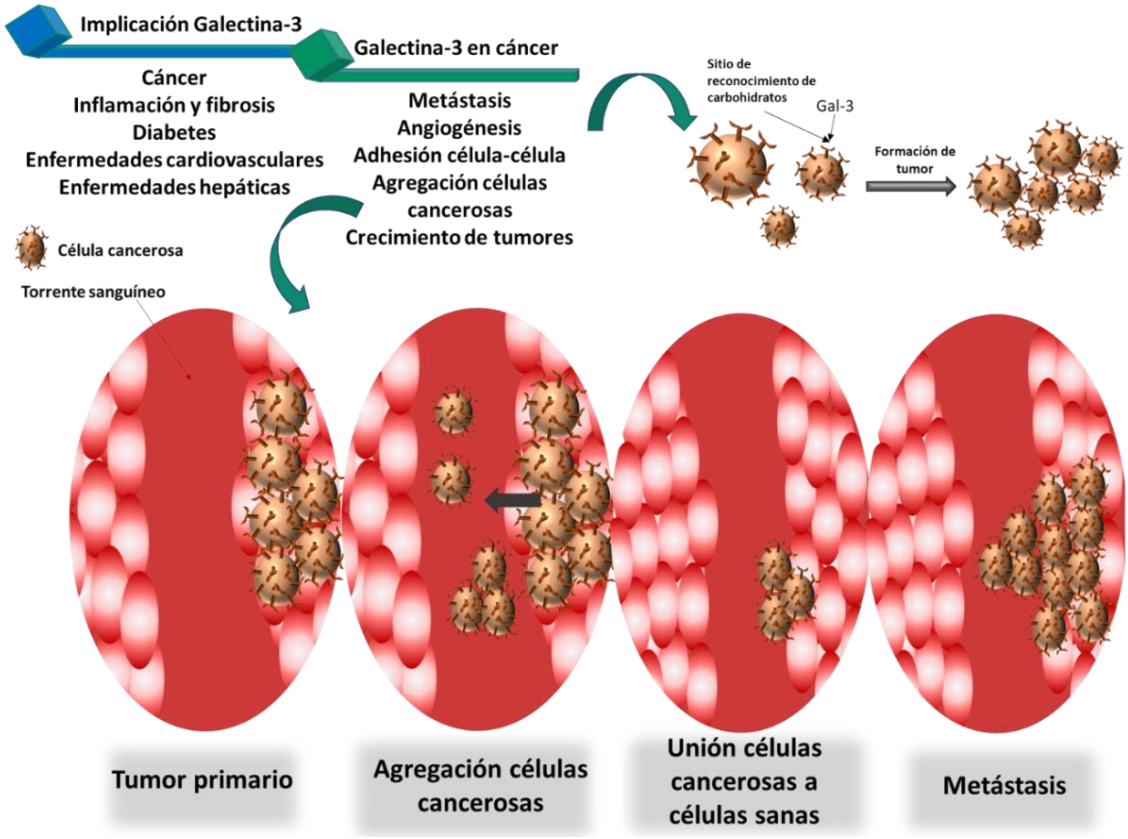

Figura 18. Implicación, formación de tumor, agregación, unión a células sanas y metástasis mediado por galectina-3

Multitud de evidencias indican que esta unión PM-Gal3 impediría la interacción de esta proteína antiapoptótica con otras proteínas y péptidos, inhibiendo así su capacidad de promover la adhesión y migración celular y promoviendo en consecuencia la apoptosis (**Morris y col., 2013; Zhang y col., 2015**) (**Fig. 19**). Estos efectos inhibidores han suscitado la posibilidad de considerar a las PMs como una tentativa potencialmente segura y no tóxica, para prevenir y reducir la carcinogénesis (**Maxwell y col., 2012**). Sin embargo, aunque se han otorgado numerosas patentes para producir PMs, es todavía difícil relacionar una estructura molecular definida con efectos bioactivos específicos. Diversas razones, entre las que se incluye la complejidad estructural de la pectina en sí, hacen difícil su caracterización. Por otra parte, aunque se ha hecho mucho trabajo en este sentido, las características específicas responsables de la unión a Gal-3 así como los mecanismos fundamentales que conducen a la actividad anticancerígena, están aún pendientes de resolverse. Sin embargo, a pesar de estas complicaciones, sí parece que son importantes cadenas de azúcares neutros principalmente arabinosa y galactosa unidas al poligalacturano (**Maxwell y col., 2012**) ya que Gal-3 Gal-3 presenta un dominio de reconocimiento de carbohidrato ~ 14 kDa (CRD). Otra de las limitaciones a la hora de entender cómo las PM ejercen tales efectos beneficiosos, es la falta de evidencias que muestren que estos carbohidratos potencialmente activos sean absorbidos tras su consumo oral. Algunas evidencias indican que un bajo grado de polimerización facilitaría la absorción intestinal de estas moléculas (**Maxwell y col., 2012**). Así, **Courts**, (2013), mostraron por primera vez, utilizando células Caco-2 como modelo de absorción in vitro (sistemas transwell), que galactanos y arabinogalactanos con bajo grado de polimerización eran absorbidos en el epitelio intestinal siguiendo una ruta de transporte paracelular, mientras que aquellos polímeros ácidos como los poligalacturónidos apenas eran absorbidos en este modelo. Así, su biodisponibilidad oral sería otro aspecto a tener en cuenta a la hora de considerar estas moléculas como potencialmente bioactivas.

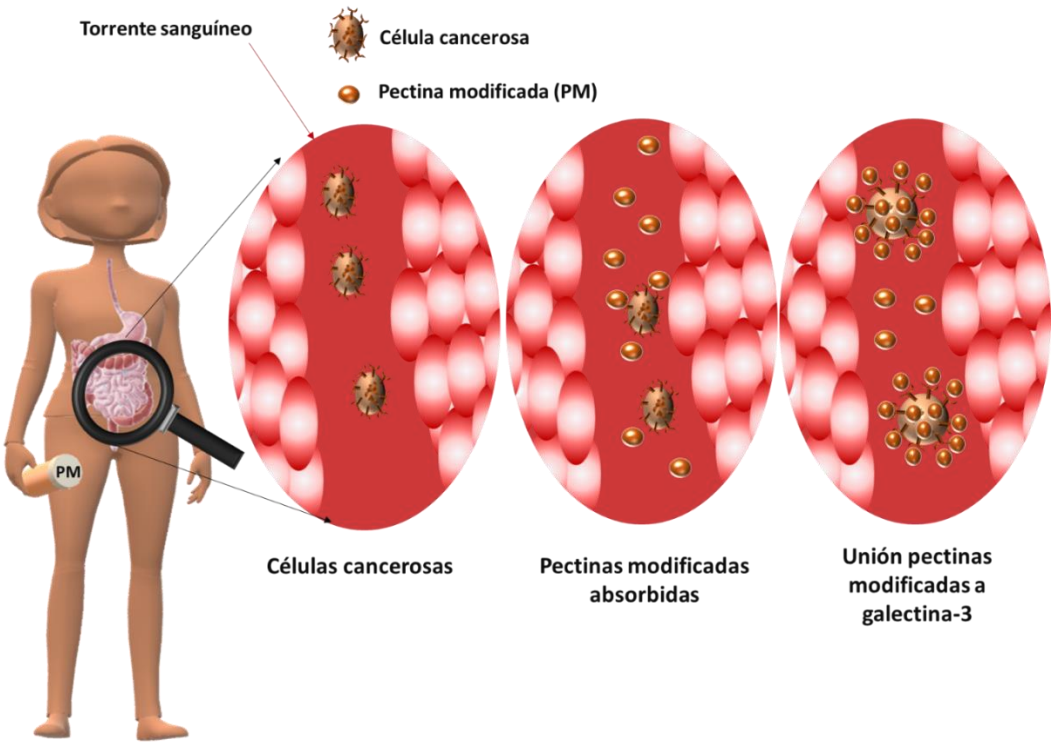

Figura 19. Representación de absorción de pectinas modificadas y su unión a células cancerosas.

3. Bibliografía

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Akahani, S., Nangia-Makker, P., Inohara, H., Kim,. HR., Raz, A. (1997). Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Research, 57(23*), 5272-6.

Allouche, N., Fki, I., Sayadi, S. 2004 Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. *Journal of Agricultural and Food Chemistry, 52*, 267–273.

Arranz, S., Silván, J.M., Saura-Calixto, F. 2010. Non extractable polyphenols, usually ignored are the mayor part of dietary polyphenols: a study on the Spanish diet. *Molecular Nutrition and Food Research, 81*, 230-242

Artajo, L.S., Romero, M.P., Suárez, M., Motilva, M.J. 2007. Partition of phenolic compounds during the virgin olive oil industrial extraction process. *European Food Research and Technogy, 225*, 617–625.

Aruoma, O.I .1998a. Free radicals, oxidative stress, and antioxidants in human health and disease. J Am Oil Chem Soc 75:199–212.
Aruoma, O.I. 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research, 523*-524, 9-20.

Aspinall, G.O. 1983. Classification of polysaccharides. En: The Polysaccharides 2. Aspinall, G.O. Ed. Academic Press. New York. 1-9.

Aunon-Calles, D., Canut, L., Visioli, F. 2013a. Toxicological evaluation of pure hydroxytyrosol. *Food and Chemical Toxicology, 55*, 498-504.

Aunon-Calles, D., Giordano, E., Bohnenberger, S., Visioli, F. 2013b. Hydroxytyrosol is not genotoxic *in vitro.* Pharmacological Research, 74 (2013a), pp. 87-93.

Baldwin, E.A., Nisperos-Carriedo, M.O., Baker, R.A. 1995. Edible coatings for lightly processed fruits and vegetables. *HortScience, 30*, 35-38.

Barba, F. J., Grimi, N., Vorobiev, E. 2014. New approaches for the use of nonconventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae. *Food Engineering Reviews, 7(1*), 45e62.

Bazzano, L.A., He, J., Orden, L.G., Loria, C.M., Whelton, P. K. 2003. Dietary fibre intake and reduced risk of coronary heart disease in US men and women: The national health

and nutrition examination survey I epidemiologic follow-up study. *Archives of Internal Medicine, 163*, 1897-1904.

Bernini, R., Merendino, N., Romani, A., Velotti, F.2013. Naturally occurring hydroxytyrosol: synthesis and anticancer potential. *Current Medicinal Chemistry, 20*, 655-670

Bianchi, G. 2003. Lipids and phenols in table olives. *European Journal of Lipid Science and Technology, 105*, 229–242

Bigagli, E., Cinci, L., Paccosi, S., Parenti, A., D'Ambrosio, M., Luceri, C. 2017. Nutritionally relevant concentrations of resveratrol and hydroxytyrosol mitigate oxidative burst of human granulocytes and monocytes and the production of pro-inflammatory mediators in LPS-stimulated RAW264.7 macrophages. *International Immunopharmacology, 43*, 147–155.

Blaut, M. 2002. Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition, 41*, 11-16.

Brett, C., & Waldron, K. 1990. Physiology and biochemistry of plant cell wall. Ed. Black, M. y Chapman, J. Unwin Hyman Boston.

Buckland, G., Gonzalez, C.A. 2015. The role of olive oil in disease prevention: A focus on the recent epidemiological evidence from cohort studies and dietary intervention trials. *The British Journal of Nutrition, 113*, S94–S101.

Burey, P., Bhandari, B.R., Howes, T., Gidley, M.J. 2008. Hydrocolloid gel particles: Formation, characterization, and application. CriticaL Reviews in Food SciencE And Nutrition, 48, 361-377.

Burgain, C., Gaiani, C., Linder, M., Scher, J. 2011. Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering, 104,* 467–483.

Campos, C. A., Gerschenson, L. N., Flores, S. K. 2010. Development of edible films and coatings with antimicrobial activity. *Food and Bioprocess Technology, 4,* 849–875.

Canesin G, Gonzalez-Peramato P, Palou J, Urrutia M, Cordón-Cardo C, Sánchez-Carbayo. 2010. M. Galectin-3 expresión is associated with bladder cancer progression and clínical outcome. Tumor Biology*, 31*, 277-85.

Capasso, R., Evidente, A., volio, S., Solla, F. 1999. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. *Journal of Agricultural and Food Chemistry, 47*, 1745-1748.

Cardoso, S.M., Silva, A.M.S., Coimbra, M.A. 2003. Structural characterisation of the olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Research, 337*, 917–924.

Carr, TP., Gallaher, D.D., Yang, C.H., Hassel, C.A. 1996. Increased intestinal contents viscosity reduces cholesterol absorption efficiency in hamsters fed hydroxypropyl methylcellulose. *Journal of Nutrition, 126*, 1463-9.

Casas, R., Sacanella, E., Estruch, R. 2014. The Immune Protective Effect of the Mediterranean Diet against Chronic Low-grade Inflammatory Diseases. Endocrine, Metabolic & Immune Disorders - Drug Targets, 14, 245-254245.

Catalan, U., Lopez de Las Hazas, M. C., Rubio, L.,... Sola, R. 2015. Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. *Molecular Nutrition & Food Research, 59*, 2523–2536.

Chan, L.W., Lee, H.Y., Heng, P.W.S. 2006. Mechanisms of external and internal gelation and their impact on the functions of alginate as a coat and delivery system. *Carbohydrate Polymer, 63*, 176-187.

Chan, S. Y., & Choo, W. S. 2013. Effect of extraction conditions on the yield and chemical properties of pectin from cocoa husks. *Food Chemistry, 141*, 3752–3758.

Chanioti, S., & Tzia, C. 2018. Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. *Innovative Food Science & Emerging Technologies, 48*, 228-239.

Chien Yie W., “Novel Drug Delivery Systems”, Second Edition, Revised and Explanded, New York, (1992).

Christian, M.S., Sharper, V.A., Hoberman, A.M.,..., R. Crea. 2004. The toxicity profile of hydrolyzed aqueous olive pulp extract**.** *Drug and Chemical toxicology, 27*, 309-330

Cicerale, S., Lucas, L., Keast, R. 2010. Biological Activities of Phenolic Compounds Present in Virgin Olive Oil *International Journal of Molecular Sciences 11*, 458-479.

Coimbra, M.A., Cardoso, S.M. Lopes-da-Silva, J.A. 2010. Olive Pomace, a Source for Valuable Arabinan-Rich Pectic Polysaccharides. *Topics in Current Chemistry 294*:129-41.

Coimbra, M.A., Waldron, K.W., Selvendran, R.R. 1994. Isolation and characterisation of cell wall polymers from olive pulp (Olea europea L.). *Carbohydrate Research, 25*2, 245-262.

Coimbra, M.A., Waldron, K.W., Selvendran, R.R. 1995. Isolation and characterisation of cell wall polymers from olive pulp (Olea europea) seed hull. *Carbohydrate Polymers, 27*, 285-294.

Comunian, T.A., Favaro-Trindade, C.S. 2016. Microencapsulation using biopolymers as an alternative to produce food enhanced with phytosterol and omega-3 fatty acids: a review. *Food Hydrocoll, 61*, 442-457.

Conti, S., Vexler, A., Hagoel, L., …. Lev-Ari, S.2018. Modified citrus pectin as a potential sensitizer for radiotherapy in prostate cancer. *Integrative cancer therapies, 17,* 1225-1234

Courts, F.L. 2013. Profiling of modified citrus pectin oligosaccharide transport across Caco-2 cell monolayers. *PharmaNutrition, 1(1),*22–31

Covas, M.I., Ruiz-Gutiérrez, V., de la Torre, R., …, Visioli, F. 2006. Minor Components of Olive Oil: Evidence to Date of Health Benefits in Humans. *Nutrition Reviews, 64*, 20- 30.

Crea, R. 2002. Method of obtaining a hydroxytyrosol-rich composition from vegetation water. United States Patent , US 6,416,808 B1.

Das, S., & Ng, K. Y. 2010. Colon-specify of reveratrol: Optimization of multi-particulate calcium-pectinate carrier. *International Journal of Pharmaceutics, 385*, 20–28.

De la Casa, J.A., Romero, I., Jiménez, J., Castro E. 2012. Fired clay masonry units production incorporating two-phase olive mill waste (alperujo). *Ceramics International, 38,* 5027–5037.

De Martino, A., Arienzo, M., Iorio, M., Vinale, F., Lorito, M., Prenzler, P.D., Ryan, D., Obied, H.K. 2011. Detoxification of olive mill wastewaters by zinc–aluminium layered double hydroxides. *Applies Clay Science, 53*, 737–744.

de Oliveira, C. F., Giordani, D., Gurak, P. D., Cladera-Olivera, F., Marczak, L. D. F. 2015. Extraction of pectin from passion fruit peel using moderate electric field and conventional heating extraction methods. *Innovative Food Science & Emerging Technologies, 29*, 201–208.

de Oliveira, C., Giordani, D., Lutckemier, R., Gurak, P. D., Cladera-Olivera, F., & Ferreira Marczak, L. D. 2016. Extraction of pectin from passion fruit peel assisted by ultrasound. *LWT - Food Science and Technology, 71*, 110–115.

de Wit, N., Esser, D., Siebelink, E., Fischer, A., Sieg, J., & Mes, J. 2019. Extrinsic wheat fibre consumption enhances faecal bulk and stool frequency; a randomized controlled trial. *Food & function 10*, 646.

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., Michaud, P. 2013. Olive mil wastes: Biochemical characterizations and valorisation strategies. *Process Biochemistry, 48*, 1532–1552.

Donhowe, G., & Fennema, O. 1993.Water vapor and oxygen permeability of wax films. *Journal of the American Oil Chemists' Society, 70*, 867–873.

Duff, S.J.B., & Murray, W.D. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. *Bioresource Technology, 55*, 1-33.

Eastwood, M.A., & Hamilton, D. 1968. Studies of the adsorption of bile salts to nonabsorbed components of diet. *Biochemical et Biophysica Acta, 152*, 165-173

Echeverría, F., Ortiz, M., Valenzuela, R., Videla L. A. 2017. Hydroxytyrosol and cytoprotection: A projection for clinical interventions. *International Journal of Molecular Sciences*, 180, 930.

Eilami, O., Oliverio, M., Hosseinian, S., Motlagh, A.H, Naghmachi, M. 2015. Antimicrobial effects of hydroxytyrosol extracted from olive leaves, on propionibacterium acnes. *Clinical Research & Methods, 15*, 187-191.

European Food Safety Authority (2011). EFSA Journal, 9, 2033 (http://www.efsa.europa.eu/ en/efsajournal/pub/2033.htm)

Fabiani, R., De Bartolomeo, A., Rosignoli, P ., Servili, M., Montedoro, G. F., Morozzi, G. 2002. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *European Journal of Cancer Prevention, 11,* 351-358.

Fang, T., Liu, D., Ning, H., Dan Liu, Sun, J., Huang, X., … Huang, R. 2018. Modified citrus pectin inhibited bladder tumor growth through downregulation of galectin-3. *Acta Pharmacologica Sinica, 39,* 1885-1893.

Farràs, M., Valls, R. M., Fernández–Castillejo, S., Giralt, M., Solà, R., Subirana, I.,… Fitó, M. 2013. Olive oil polyphenols enhance the expression of cholesterol efflux related genes in vivo in humans. A randomized controlled trial. *Journal of Nutritional Biochemistry, 24*, 1334–1339.

Felizón, B., Fernández-Bolaños, J., Heredia, A., Guillén, R. 2000. Steam-explosion pretreatment of olive cake. *Journal of the American Oil Chemist's Society, 77*, 15-22.

Ferguson, L.R., Harris, P.J., 1996 Studies on the role of specific dietary fibres in protection against colorectal cancer. *Mutation research, 19*, 173-84.

Fernández-Bolaños Guzmán, J., Heredia Moreno, A., Rodríguez Gutiérrez, G., Rodríguez Arcos, R., Jiménez Araujo, A., Guillén Bejarano, R. 2002b. Mejoras introducidas en la patente principal nº 200002422 relativa a un procedimiento de obtención de hidroxitirosol purificado a partir de productos y subproductos derivados del olivo. ES 2 177 457 A1.

Fernandez-Bolanos, J Rodriguez, G Rodriguez, R Guillen, R Jimenez, A. 2006. Extraction of interesting organic compounds from olive oil waste. *Grasas & Aceites, 57*, 95-106

Fernández-Bolaños, J., Felizón B., Heredia, A., Rodríguez, R., Guillén, R., Jiménez, A. 2001. Steam-explosion of olive stones: hemicellulose solubilization and enhancement of enzymatic hydrolysis of cellulose. *Bioresource Technology, 79*, 53-61.

Fernández-Bolaños, J., Felizón, B., Brenes, M., Guillén, R., Heredia A. 1998. Hidroxitirosol and Tyrosol as the Main Compounds Found in the Phenolic Fraction of Steam- Exploded Olive Stones. *Journal of the American Oil Chemists' Society*, *75,* 1643-1649.

Fernández-Bolaños, J., Rodríguez Gutiérrez, G., Lama Muñoz, A., Sánchez Moral, P. 2012. Device and method for processing olive-oil-production byproducts. Patent nº WO2012020159

Fernández-Bolaños, J., Rodríguez, G., Lama-Muñoz, A., Rubio-Senent, F., Fernández-Bolaños, J.M.G., Castilla-Maya, I., López-López, O., Castro-Marset, A. 2013. Procedimiento para la obtención de extracto de hidroxitirosol, extracto mezcla de hidroxitirosol y 3,4-dihidroxifenilglicol, y extracto de acetato de hidroxitirosilo, a partir de subproductos del olivo, y su purificación. WO213/007850 A1.

Fernández-Bolaños, J., Rodríguez, G., Rodríguez, R., Heredia, A., Guillén, R., Jiménez, A. 2002a. Production in large quantities of highly purified hidroxitirosol from liquid-solid waste of two-phase olive oil processing or "alperujo". *Journal of Agricultural and Food Chemistry, 50*, 6804-6811

Ferri, F., Bertin, L., Scoma, A., Marchetti, L., Fava, F. 2011 Recovery of low molecular weight phenols through solid-phase extraction. *Chemical Engineering Journal, 166*, 994–1001

Fitó, M., Cladellas, M., de la Torre, R., Marti, J., Munoz, D., Schroder, H., ... Covas, M. I. 2008. Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: A randomized crossover controlled trial*. European Journal of Clinical Nutrition, 62(*4), 570–574

Forsman, H., Islander, U., Andreasson, E., Andersson, A., Onnheim, K., Karlstrom, A., Sävman, K., Magnusson, M., Brown, K.L., Karlsson, A. 2011. Galectin-3 aggravates joint inflammation and destruction in antigen-induced arthritis. *Arthritis Rheumatism, 63*, 445-454.

Frascari, D., Bacca, A.E.M., Zama, F., Bertin, L., Fava, F., & Pinelli, D. 2016 Olive mill wastewater Valorisation through phenolic compounds adsorption in a continuous flow column. *Chemical Engineering Journal 283*, 293–303.

Fuccelli R., Fabiani R., Rosignoli P. 2018. Hydroxytyrosol Exerts Anti-Inflammatory and Anti-Oxidant Activities in a Mouse Model of Systemic Inflammation. *Molecules, 5*;23(12).

Galanakis, C.M., Tornberg, E., Gekas, V. 2010. The effect of heat processing on the functional properties of pectin contained in olive mill wastewater. *LWT – Food Science and Technology 43*, 1001–1008.

Galus, S., & Kadzińska, J. 2015. Food applications of emulsion-based edible films and coatings. *Trends in Food Science & Technology, 45*, 273–283

Gao, X., Zhi, Y., Sun, L., Peng, X., Zhang, T., Xue, H., Tai, G., Zhou, Y. 2013. The Inhibitory effects of a rhamnogalacturonan I (RG-I) domain from ginseng pectin on galectin-3 and its structure-activity relationship. *Journal of Biological Chemistry, 288* (47), 33953–33965.

García MA, Martino MN, Zaritzky N. 1998. Starch-based coatings: effect on refrigerated strawberry (Fragaria ananassa) quality. *Journal Food and Agricultural 76*, 411-420.

García, A., Rodríguez-Juan, E., Rodríguez-Gutiérrez, G., Rios J.J., Fernández-Bolaños, J. 2016. Extraction of phenolic compounds from virgin olive oil by deep eutectic solvents (DESs). *Food Chemistry, 15*, 554-61.

Gennadios, A. y Weller, C. L. 1990. Edible films coating from wheat and corn protein. *Food Technology, 44*, 63- 69.

Ghalandari, M., Naghmachi, M., Oliverio, M.,… Eilami, O. 2018. Antimicrobial effect of hydroxytyrosol, Hydroxytyrosol Acetate and Hydroxytyrosol Oleate on Staphylococcus Aureus and Staphylococcus Epidermidis. *Electronic Journal of General Medicine, 5*(4).
Ghanbari, R., Anwar, F., Alkharfy, K.M., Gilani, A.-H., Saari, N. 2012. Valuable Nutrientsand Functional Bioactives in Different Parts of Olive (Olea europaea L.)—A Review. *International Journal of Molecular Science, 13*, 3291-3340.

Gibson, G. R. & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota-introducing the concept of prebiotics. *Journal of Nutrition*, *125*, 1401-1412.

Glinsky, V.V, & Raz A. 2009. Modified citrus pectin anti-metastatic properties: one bullet, multiple targets. Carbohydrate *Research, 344,* 1788–91.

Granados-Principal, S., El-Azem, N., Pamplona R.,…., Ramirez-Tortosa M. 2014. Hydroxytyrosol ameliorates oxidative stress and mitochondrial dysfunction in

doxorubicin-induced cardiotoxicity in rats with breast cancer. *Biochemical pharmacology, 1*;90, 25-33.

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Ochoa-Herrera, J., Perez-Lopez, P., Pulido-Moran, M., Ramirez-Tortosa, M. C. 2012. Squalene ameliorates atherosclerotic lesions through the reduction of CD36 scavenger receptor expression in macrophages. *Molecular Nutrition & Food Research, 56*(5), 733–740.

Grous, J.J., Redfern, C.H., Mahadevan, D., Schlinder, J. 2006. GCS-100, a galectin-3 antagonist, in refractory solid tumors: a phase I study. Journal of Clinical Oncology, ASCO. Annual Meeting Proceedings part I, 24 (18S).

Guarner, F. 2003. Gut flora in health and disease. *The Lancet, 360*, 512-19.

Guimarães, A., Abrunhosa, L., Pastrana, L.M., Cerqueira, M.A. 2018. Edible Films and Coatings as Carriers of Living Microorganisms: A New Strategy Towards Biopreservation and Healthier Foods

Gutiérrez, T.J. 2017. Surface and nutraceutical properties of edible films made from starchy sources with and without added blackberry pulp. *Carbohydrate Polymers, 165* 169–179.

Harris, P.J. 1990. Plant cell wall structure and development. En: Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Rumiants. Alkin, E. D. (ed.). Elservier Sci. Publ. Co. Nueva York, pp. 71-90

Hatfield, R.D. 1993. Cell wall polysaccharide interaction and degradability. En: Forage Cell Wall Structure and Digestibility. Ed. Jung, H. G., Buxton, D. R., Hatfield, D. R., Ralph, J. ASA/CSSA/SSSA. Madison, Wisconsin, USA. 285-313

Heredia, A., Guillén, R., Jiménez, A., Fernández-Bolaños, J. 1993. Plant cell Wall structure. *Revista Española de Ciencia y Tecnología de Alimentos, 33*, 113-131.

Heredia, A., Jiménez, A., Guillén, R. 1995. Composition of plant cell wall. Z *Lebensm. Uniter Forsch, 200,* 24-31

Herrero, M., Temirzoda, T. N., Segura-Carretero, A., Quirantes, R., Plaza, M., Ibañez, E. (2011). New possibilities for the valorization of olive oil by-products. *Journal of Chromatography A, 1218(42)*, 7511e7520.

Honjo, Y., Nangia-Makker, P., Inohara, H., Raz, A. 2001. Down-regulation of galectin-3 suppresses tumorigenicity of human breast carcinoma cells. Clinical cancer research : an official journal of the *American Association for Cancer Research, 7(3*), 661-8.

Hosseini, S. S., Khodaiyan, F., & Yarmand, M. S. 2016. Aqueous extraction of pectin from sour orange peel and its preliminary physicochemical properties. *International Journal of Biological Macromolecules, 82,* 920–926.

Hsu, C.-K., Liao, J.-W., Chung, Y.-C., Hsieh, C.-P., Chan, Y.-C. 2004. Xylooligosaccharides and Fructooligosaccharides Affect the Intestinal Microbiota and Precancerous Colonic Lesion Development in Rats. *American Society for Nutritional Science,* 1523-1528.

Iiyama, K., Lam, T.B., Stone, B.A. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemestry, 29*, 733-737

Jackson, C. L,; Dreaden, T. M., Theobald, L. K.,… Mohnen, D. 2007. Pectin Induces Apoptosis in Human Prostate Cancer Cells: Correlation of Apoptotic Function with Pectin Structure. *Glycobiology*, *17* (8), 805–819.

Jane, M., McKay, J., Pal, S. 2018. Effects of daily consumption of psyllium, oat bran and polyGlycopleX on obesity-related disease risk factors: A critical review. *Nutrition 57*, 84-91.

Jiménez, A. 1993. Modificación de la pared celular de aceitunas durante su elaboración. *Tesis doctoral*.

Jiménez, A., Guillén, R., Fernández-Bolaños, J., Heredia, A. 1994. Cell wall composition of olives. *Journal of Food Science, 59*, 1192-1196.

Jun Tao, Sha Li, Ren-You Gan, Cai-Ning Zhao, Xiao Meng & Hua-Bin Li. 2019. Targeting gut microbiota with dietary components on cancer: Effects and potential mechanisms of action. *Critical Reviews in Food Science and Nutrition.* https://doi.org/10.1080/10408398.2018.1555789.

Jung, H.G., & Deetz, D.A. 1993. Cell wall structure and digestibility. En: Forage Cell Wall Structural and Digestibility. Ed. Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J. ASA/CSSA/SSSA. Inc. Madison, Wisconsin, USA. 315-346

Kalogerakis, N., Polito, M., Foteinis, S., Chatzisymeon, E., Mantzavinos, D. 2013. Recovery of antioxidants from olive mill wastewaters: A viable solution that promotes their overall sustainable management. *Journal of Environmental Management, 128*, 749-758.

Kester J.J, & Fennema, O.1986. Edible films and coatings: a review. Food Technol. 40:47-59.

Khazaei, N., Esmaiili, M., Emam-Djomeh, Z. 2016. Effect of active edible coatings made by basil seed gum and thymol on oil uptake and oxidation in shrimp during deep-fat frying. *Carbohydrate Polymers 137*, 249–254.

Killeen, M. J., Linder, M., Pontoniere, P., & Crea, R. 2014. NF-kappabeta signaling and chronic inflammatory diseases: Exploring the potential of natural products to drive new therapeutic opportunities. Drug Discovery Today, 19(4), 373–378

Kyriazis, J. D., Aligiannis, N., Polychronopoulos, P., Skaltsounis, A. L., Dotsika, E. 2013. Leishmanicidal activity assessment of olive tree extracts. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology, 20*(3–4), 275–281.

Lakkis, J.M. Introduction. In Encapsulation and controlled release technologies in food systems. Edited by Lakkis JM. Blackwell Publishing 2007:1-15.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Fernández-Bolaños, J. 2012. Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids, 28*, 92-104

Lama-Múñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Fernández-Bolaños, J. 2011. New hydrothermal treatment of alperujo enhances the content of bioactive minor components in crude pomace olive oil. *Journal of Agricultural and Food Chemistry, 59*, 1115- 1123

Lefsih, K., Giacomazza, D., Dahmoune, F., Mangione, M. R., Bulone, D., San Biagio, P. L.,... Madani, K. 2017. Pectin from Opuntia ficus indica: Optimization of microwaveassisted extraction and preliminary characterization. *Food Chemistry, 221*, 91– 99.

Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J-C., Lorquin, J., Delattre, M., Simon, J-L., Asther, M., Labat, M. 2001. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chemistry, 75*, 501-507.

Li, C.L., Martini, L.G., Ford, J.L., Roberts, M. 2005. The use of hypromellose in oral drug delivery. Journal of *Pharmacy* and *Pharmacology, 57*, 533-546.

Li, Y., Liu, L., Niu, Y., Feng, J., Sun, Y., Kong, X., Chen, Y., Chen, X., Gan, H., Cao, S., Mei, Q. 2012. Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitisassociated colon cancer: role of galectin-3 and apoptosis in cancer prevention. European journal of nutrition, *51*, 107-17.

Liu, D., & Wang, M. (2010ver años) Novel use of hydroxytyrosol and olive extracts/concentrates containing in it. Patent US0130620 A1.

Liu, D., Martinez-Sanz, M., Lopez-Sanchez, P., Gilbert, E. P., Gidley, M. J. 2017. Adsorption behaviour of polyphenols on cellulose is affected by processing history. *Food Hydrocolloids*, *63*, 496–507.

Liyama, K., Lam, T.B., Stone, B.A. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemestry, 29*, 733-737.

López de Las Hazas, M. C., Piñol, C., Macià, A., & Motilva, M. J. (2017). Hydroxytyrosol and the colonic metabolites derived from virgin olive oil intake induce cell cycle arrest and apoptosis in colon cancer cells. Journal of Agricultural and Food Chemistry, 19.

López-Oliva, M.E., Agis-Torres, A., Goñi, I., Muñoz-Martínez, E. 2010. Grape antioxidant dietary fibre reduced apoptosis and induced a pro-reducing shift in the glutathione redox state of the rat proximal colonia mucosa. *British Journal of Nutrition, 103*, 1110-1117.

Maestrelli, F., Zerrouk, N., Cirri, M., Mennini, N., Mura, P. 2008. Microspheres for colonic delivery of ketoprofen-hydroxypropyl–cyclodextrin complex. *European* Journal of *Pharmaceutical* Sciences, *34*, 1-11.

Makki, K., Deehan, E.C., Walter, J., Bäckhed, F. 2018. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. *Cell host & microbe. 13;23*, 705-715

Makris, D.P 2018 Green extraction processes for the efficient recovery of bioactive polyphenols from wine industry solid wastes - Recent progress. *In Green And Sustainable Chemistry, 13*, 50- 55.

Malapert, A., Reboul, E., Loonis, M., Dangles, O., Tomao, V., 2018. Direct and rapid profiling of biophenols in olive pomace by UHPLC-DAD-MS. *Food Analytical Methods 11*, 1001–1010.

Maneerat, N., Tangsuphoom, N., & Nitithamyong, A. 2017. Effect of extraction condition on properties of pectin from banana peels and its function as fat replacer in salad cream. *Journal of Food Science and Technology, 54*, 386–397.

Markov, P.A., Popov, S.V., Nikitina, I.R., Ovodova, R.G., Ovodov, Y.S. 2011. Anti-inflammatory activity of pectins and their galacturonan backbone. *Russian Journal of Bioorganic Chemistry, 37*, 817-821.

Martínez–Martos, J. M., Mayas, M. D., Carrera, P., de Saavedra, J. M. A., Sánchez–Agesta, R., Arrazola, M., Ramírez-Expósito, M.J. 2014. Phenolic compounds oleuropein and hydroxytyrosol exert differential effects on glioma development via antioxidant defense systems. *Journal of Functional Foods, 11,* 221–234.

Mateos, R., Martínez-López, S., Arévalo, G. B., Amigo-Benavent, M., Sarriá, B., & Bravo-Clemente, L. 2016. Hydroxytyrosol in functional hydroxytyrosol-enriched biscuits is highly

bioavailable and decreases oxidised low density lipoprotein levels in humans. *Food Chemistry, 205*, 248–256

Maxwell, E. G., Colquhoun, I. J., Chau, H. K., Hotchkiss, A. T., Waldron, K. W., Morris, V. J., Belshaw, N.J. 2015. Rhamnogalacturonan i containing homogalacturonan inhibits colon cancer cell proliferation by decreasing ICAM1 expression. *Carbohydrate Polymers, 132,* 546–553.

Maxwell, E.G., Belshaw, N.J., Waldron, K.W., Morris, V.J. 2012. Pectin - an emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24* (2) 64-73

May, C. D. 1990. Industrial pectins: Sources, production and applications. *Carbohydrate Polymers, 12*(1), 79–99.

McHugh, T.H y Krochta, J.M. 1994a. Sorbitol vs glycerol plasticized whey protein ediblebfilms: integrated oxygen permeability and tensile property evaluation. *Journal of Agricultural and Food Chemistry, 42*, 841-850.

McHugh, T.H y Krochta, J.M. 1994b. Milk-protein-based edible films and coatings*. Food Technology, 48,* 97-103.

McHugh, T.H. 2000. Protein-lipid interactions in edible films and coatings*. Nahrung. 44*, 148-51

Metzler B. & Mosenthin, R. 2008. A review of interactions between dietary fiber and the gastrointestinal microbiota and their consequences on intestinal phosphorus metabolism in growing pigs. *Asian-Australasian Journal of Animal Sciences. 21(4),* 603-615.

Mitchell, H., & Tiihonen, K. 2003. Prebiotics. *NutraCos,* 20, 24.

Mok, W.S.-L., & Antal, M.J.A., 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. *Industrial & Engineering Chemistry Research, 31*, 1157– 1161.

Monties, B. 1980. Les polymers vegetaux. Polymères pariétaux et alimentaires nonazotes. Collection Biochinie Appliqué. Ed. Gauthier-Villars, París.

Morris, V. J., Belshaw, N. J., Waldron, K. W., & Maxwell, E. G. 2013. The bioactivity of modified pectin fragments. *Bioactive Carbohydrates and Dietary Fibre*, 1, 21-37.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladish, M. 2005. Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresource Technology, 96,* 673-686.

Mussatto, S.I., Mancilha, I.M. 2007. Non-digestible oligosaccharides: A review. *Carbohydrate Polymers, 68*, 587-597.

Nangia-Makker, P., Nakahara, S., Hogan, V., Raz, A. 2007. Galectin-3 in apoptosis, a novel therapeutic target. *Journal of Bioenergetics And Biomembranes, 39*, 79-84.

Navarro, M., & Morales, F. J. 2015. Mechanism of reactive carbonyl species trapping by hydroxytyrosol under simulated physiological conditions. *Food Chemistry, 175*,92–99.

Nísperos-Carriedo., M.O. 1994. Edible coatings and films based on polysaccharides. In: Edible coatings and films to improve food quality. JM Krochta, EA Baldwin, M Nisperos-Carriedo (eds.). pp. 305-355. Lancaster: Technomic Publishing Co.

Nunes, A.M., Pawlowski, S., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P., Velizarov, S. 2018. Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration. *Science of the Total Environment, 20,* 40-47.

Nunes, M.A., Pimentel, F.B., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. 2016. Olive byproducts for functional and food applications: challenging opportunities to face environmental constraints. *Innovative Food Science and Emerging Technologies, 35*, 139–148.

Nussinovitch, A. 1997. Agricultural uses of hydrocolloids. In: Hydrocolloid applications: Gum technology in the food and other industries. pp. 169-189. London: Blackie Academic and Professional.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D., Robards, K., Stockmann, R. 2005. Bioactivity and analysis of biophenols recovers from olive mill waste. *Journal of Agricultural and Food Chemistry, 53*, 823-837.

Obied, H.K., Bedgood, D.R., Prenzler, P.D., Robards, K. 2007. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Analytica Chimica Acta, 603*, 176-189.

Obied, H.K., Prenzler, P.D., Robards, K. 2008. Potent antioxidant biophenols from olive mill waste. *Food Chemistry, 111*, 171–178.

Okamura, D.M., Pasichnyk, K., Lopez-Guisa, J.M., Collins, S., Hsu, D.K., Liu, F.T., Eddy, A.A. 2011. Galectin-3 preserves renal tubules and modulates extracelular matrix remodelling in progressive fibrosis. American Journal of Physiology Renal Physiology, 300, F245-F253.

Olano-Martin, E., Gibson, G. R., & Rastall, R. A. 2002. Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides. *Journal of Applied Microbiology, 93(3)*, 505–511.

Olano-Martín, E., Rimbach, G.H., Gibson, G.R., Rastall, R.A. 2003. Pectin and pecticoligosaccharides induce apoptosis *In vitro* human colonia adenocarcionoma cells. *Anticancer Research, 23*, 341-346.

Padayachee, A., Day, L., Howell, K., Gidley, M. J. 2017. Complexity and health functionality of plant cell wall fibers from fruits and vegetables. *Critical Reviews in Food Science and Nutrition*, *57*(1), 59–81.

Parkinson, L., Cicerale, S., 2016. The health benefiting mechanisms of virgin olive oil phenoliccompounds. *Molecules, 21*, 1734.

Parra-Huertas, R.A (2010). Revisión: Microencapsulación de alimentos food microencapsulation: A review. *Revista Facultad Nacional de Agronomía, Medellin, 63*, 5669-5684.

Patil, J.S., Kamalapur, M.V., Marapur, S.C., Kadam, D.V. 2010. Ionotropic gelation and polyelectrolyte complexation: the novel technique to design hydrogel particulate sustained, modulated drug delivery system: a review. Digest *Journal of Nanomaterials* and *Biostructures*, 5, 241-248.

Pérez-Gago, M., & Krochta, J. 2002. Drying temperature effect on water vapour permeability and mechanical properties of whey protein-lipid emulsion films. *Journal of Agricultural and Food Chemistry, 49*, 2308-2312.

Pérez-Jiménez, F., Ruano, J., Perez-Martinez, P., Lopez-Segura, F., Lopez-Miranda, J. 2007. *Molecular nutrition & food research.51,* 1199-1208

Pérez-Jiménez, J., & Saura-Calixto, F. 2015. Macromolecular antioxidants or non-extractable polyphenols in fruit and vegetables: Intake in four European countries. *Food Research International, 74*, 315–323.

Pérez-Jiménez, J., & Saura-Calixto, F. 2018. Fruit peels as sources of non-extractable polyphenols or macromolecular antioxidants: Analysis and nutritional implications. *Food Research International, 111*, 148–152.

Popov, S. V., Markov, P. A., Popova, G. Y., Nikitina, I. R., Efimova, L., Ovodov, Y. S. 2013. Anti-inflammatory activity of low and high methoxylated citruspectins. *Biomedicine & Preventive Nutrition, 3*, 59–63.

Procopio A, Alcaro S, Nardi M, Oliverio M, Ortuso F, Sacchetta P, Pieragostino D, Sindona G. 2009. Synthesis, Biological Evalutation, and Molecular Modeling of Oleuropein and Its Semisynthetic Derivatives as Cyclooxygenase Inhibitors. *Journal Agricultural & Food Chemistry, 57*, 11161–11167

Radosavljevic, G., Jovanovic, I., Majstorovic, I.,… Jonjic, S., Lukic, M.L. 2011. Deletion of galectin-3 in the host attenuates metastasis of murine

melanoma by modulating tumor adhesion and NK cell activity. *Clinical & experimental metastasis., 28(5*), 451-62.

Ramírez-Expósito, M.J., & Martínez-Martos, J.M. 2018. Anti-Inflammatory and Antitumor Effects of Hydroxytyrosol but Not Oleuropein on Experimental Glioma In Vivo. A Putative Role for the Renin-Angiotensin System. *Biomedicines, 6*, 11.

Ramos, L.P. 2003. The chemistry involved in the steam treatment of lignocellulosic materials. *Quimica Nova, 26,* 863-871.

Raz, A., & Pienta, K.J. 1998. Method for inhibiting cancer metastasis by oral administration of solubile modified citrus pectin, US Patent nº 5834442.

Reboredo-Rodríguez, P., Figueiredo-González,M., González-Barreiro, C., Simal-Gándara, J., Salvador, M.D., Cancho-Grande, B., Fregapane, G. 2017. State of the art on functional virgin olive oils enriched with bioactive compounds and their properties. *The International Journal of Molecular Sciences, 18*, 668

Reis, M. T. A., de Freitas, O. M. F., Ferreira, L. M., Carvalho, J. M. R. 2006. Extraction of 2-(4-hydroxyphenyl)ethanol from aqueous solution by emulsion liquid membranes. *Journal of Membrane Science, 269* , 161-170.

Robles-Almazan, M., Pulido-Moran, M., Moreno-Fernandez, J., Ramirez-Tortosa, C., Rodriguez-Garcia, C., Quiles, J. L., Ramirez-Tortosa, M. C. 2018. Hydroxytyrosol: Bioavailability, toxicity, and clinical applications. *Food Research International, 105*, 654–667.

Rodis, P.S., Karathanos, V.T., Mantzavinou, A. 2002. Partitioning of olive oil antioxidants between oil and water phase. *Journal of Agricultural and Food Chemistry, 50*, 596-601.

Rodrigues, F., Nunes, M.A., Oliveira, M.B.P.P., 2017. Chapter 12 - applications of recovered bioactive compounds in cosmetics and health care products. In: Galanakis, C.M. (Ed.), Olive Mill Waste - Recent Advances for Sustainable Management. Academic Press, pp. 255–274.

Rodríguez, G., Rodríguez, R., Fernández-Bolaños, J., Guillén, R., Jiménez, A. 2007b. Antioxidant activity of effluents during the purification of hydroxytyrosol and 3,4-dihydroxyphenylglycol from olive oil waste. *European Food Research and Technology, 224*, 733-741.

Rodríguez, G., Rodríguez, R., Jiménez, A., Guillén, R., Fernández-Bolaños, J. 2007a. Effect of steam treatment of alperujo on the composition, enzymatic saccharification, and in vitro digestibility of alperujo. *Journal of Agricultural and Food Chemistry, 55*, 136-142.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., Heredia, A. 2006. Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science and Technology, 17,* 3-15.

Rodríguez-Gutiérrez, G., Duthie, G.G., Wood, S., Morrice, P., Nicol, F., Reid, M., Cantlay, L.L., Kelder, T., Horgan, G.W., Fernández-Bolaños, J., de Roos, B. 2012. Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats--a proteomics and network analysis approach. *Molecular Nutrition* & *Food Research*, 56, 1137-47.

Rodríguez-Gutíerrez, G., Jiménez-Araujo, A., Rodríguez-Arcos, R., Lama-Muñoz, A.,Fernández-Bolaños, J., Guillén-Bejarano, R. 2011. Procedimiento de purificación de 3,4-dihidroxifenilglicol (DHFG) a partir de productos vegetales.Patente nº ES2341526 B1.

Rodríguez-Gutiérrez, G., Lama Muñoz, A., Pérez-Lanzac, M., Espartero, J.L.., Fernández-Bolaños, J. 2009. Isolation of a powerful antioxidant from Olea europaea fruit-mill waste: 3,4-Dihydroxyphenylglycol. *LWT - Food Science and Technology 42*, 483-430.

Rojas-Grau, M.A., Soliva-Fortuny, R., Martin-Belloso, O. 2009. Edible coatings to incorporate active ingredients to freshcut fruits. *Trends in Food Science and Technology, 20*, 438 – 447.

Romero, C., Brenes, M., Yousfi, K., García, P., García, A., Garrido, A. 2004. Effect of cultivar and processing method on the contents of polyphenols in table olives. *Journal of Agricultural and Food Chemistry*, *52*, 479-484.

Romero, C., García, P., Brenes, M., García, A., Garrido, A. 2002. Phenolic compounds in natural black Spanish olive varieties. European Food Research and Technology, 215, 489-496.

Rosello-Soto, E., Barba, F.J., Lorenzo, J.M., Munekata P.E.S., Gómez, B., Moltó, J.C. 2019. Phenolic profile of oils obtained from "horchata" by-products assisted by supercritical-CO2 and its relationship with antioxidant and lipid oxidation parameters: Triple TOF-LC-MS-MS characterization. *Food Chemistry, 274*, 865-871.

Roselló-Soto, E., Galanakis, C. M., Brncic, M., Orlien, V., Trujillo, F. J., Mawson, R.,…. Barba, F.J. (2015b). Clean recovery of antioxidant compounds from plant foods, byproducts and algae assisted by ultrasounds processing. Modeling approaches to optimize processing conditions. *Trends in Food Science & Technology, 42(2*), 134e149.

Roselló-Soto, E., Koubaa, M.,Moubarik, A., Lopes, R. P., Saraiva, J. A., Boussetta, N., ... Barba, F. J. (2015a). Emerging opportunities for the effective valorization of wastes and byproducts generated during olive oil production process: Non-conventional methods for the recovery of high-added value compounds. *Trends in Food Science & Technology, 45*, 296–310.

Rubio-Senent, F., de Roos, B., Duthie, G., Fernández-Bolaños, J., Rodríguez-Gutiérrez, G. 2015c. Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E-deficient rats. *European Journal of Nutrition*, 54, 1287-95

Rubio-Senent, F., Rodríguez-Gutierrez, G., Lama-Munoz, A., & Fernandez-Bolaños, J. 2015a. Pectin extracted from thermally treated olive oil by-products: Characterization, physico-chemical properties, in vitro bile acid and glucose binding. *Food Hydrocolloids, 43,* 311-321

Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Munoz, A., Garcia, A., & Fernandez-Bolaños, J. 2015b. Novel pectin present in new olive mill wastewater with similar emulsifying and better biological properties than citrus pectin. *Food Hydrocolloids, 50*, 237-246.

Ruiz-Canela, M., Estruch, R., Corella, D., Salas-Salvado, J., Martinez-Gonzalez, M.A. 2014. Association of mediterranean diet with peripheral artery disease: The PREDIMED randomized trial. *Journal of the American Medical Association, 311*-415

Ryan, D., & Robards, K. 1998. Phenolic compounds in olives. *The Analyst, 123*, 31-44.

Samuelsson, L. M., Young, W. N., Fraser, K., Tannock, G. W., Lee, J., Roy, N. C. 2016. Digestive-resistant carbohydrates affect lipid metabolism in rats. *Metabolomics, 12(5*), 1–13.

Saura-Calixto, F. 1998. Antioxidant dietary fiber product: a new concept and a potencial food ingredient. *Journal of Agricultural and Food Chemistry, 46*, 4303-4306.

Saura-Calixto, F. 2011. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. *Journal of Agricultural and Food Chemistry, 59,* 43-49.

Saura-Calixto, F., & Díaz-Rubio, M.E. 2007. Polyphenols associated with dietary Wbre in wine A wine

Seixas, F. L., Fukuda, D. L., Turbiani, F. R. B., Garcia, P. S., Petkowicz, C. L. de O., Jagadevan, S., & Gimenes, M. L. 2014. Extraction of pectin from passion fruit peel (*Passiflora edulis f. flavicarpa*) by microwave-induced heating. *Food Hydrocolloids, 38*, 186-12

Showalter, A. M. 1993. Structure and function of plant cell wall proteins. *Plant Cell, 5*, 9-23.

Silva, S., Sepodes, B., Rocha, J.,…Figueira., M.E. 2015. Protective effects of hydroxytyrosol-supplemented refined olive oil in animal models of acute inflammation and rheumatoid arthritis. *Journal of Nutritional Biochemistry*, 26, 360–368.

Sjösttröm, E. 1982. Wood chemistry. Fundamentals and Applications. Academic Press. London. pp. 15-50.

Sothornvit R, Krochta, J.M. 2000. Plasticizer effect on oxygen permeability of Blactoglobulin films. *Journal of Agricultural and Food Chemistry,* 48, 6298-6302.

Southgate, D. A. T. 1990. Determination of Carbohydrates. Elservier Applied Science Londres.

Toteda, G., Lupinacci, S., Vizza, D., Bonofiglio, R., Perri, E., Bonofiglio, M., ... Perri, A. 2017. High doses of hydroxytyrosol induce apoptosis in papillary and follicular thyroid cancer cells. *Journal of Endocrinological Investigation, 40*, 153–162.

Traini, S., Piccolo, E., Tinari, N. ….Natoli, S. 2014. Inhibition of tumor growth and angiogenesis by SP-2, an antilectin, galactoside-binding soluble 3 binding protein (LGALS3BP) antibody. *Molecular cancer therapeutics, 13*, 916–925.

Tura, D., & Robards, K. 2002. Sample handling strategies for the determination of biophenols in food and plants. *Journal of Chromatography A*, *25*, 71-93.

Valls, R. M., Farràs, M., Suárez, M.,… Solà, R. 2015. Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomised controlled trial. *Food Chemistry, 167*, 30–35.

Van Buren, J. P. 1979. The chemistry of texture in fruits and vegetables. *Journal of Texture Studies, 10,* 1-23.

Van Dooren, C., Douma, A., Aiking, H., & Vellinga, P. 2017. Proposing a novel index reflecting both climate impact and nutritional impact of food products. *Ecological Economics, 131*, 389–398.

Venneri, M. G., Del Rio, G. 2004. Systematic study of long-term stability of 3,4-dihydroxyphenylglycol in plasma for subsequent determination with liquid chromatography. *Journal of Chromatography B, 802*, 247-255.

Visioli, F., Bogani, P., Grande, S., Galli, C. 2004. Olive oil and oxidative stress. *Grasas y Aceites , 5*5, 66-75.

Vuong, L., Kouverianou, E., Rooney, C.M……., MacKinnon, A.C. 2019. An orally active galectin-3 antagonist inhibits lung adenocarcinoma growth and augments response to PD-L1 blockade. *American Association for Cancer.*

Wang, M., Huang, B., Fan, C., Zhao, K., Hu, H., Xu, X., & Liu, F. 2016. Characterization and functional properties of mango peel pectin extracted by ultrasound assisted citric acid. *International Journal of Biological Macromolecules, 91,* 794–803.

Wikiera, A., Mika, M., Starzyńska-Janiszewska, A., Stodolak, B. 2016. Endo-xylanase and endo-cellulase-assisted extraction of pectin from apple pomace. *Carbohydrate Polymers, 142*, 199–205.

Willem, J., Jones, J., MacCleary, B., Topping, D. 2010. Dietary Fibre: New frontiers for food and health. *Wageningen Academic Publisher: Wageningen, The Netherlands,* 1-586.

Wilson, J. R. 1993. Organization of forage plant tissues. Forage Cell Wall Structure and Digestability. Jung, H. G., Buxton, D. R:, Hatfield, R. D:, Ralph, J. (eds). Madison, Wisconsin, pp. 1-32.

Wilson, W.D., Jarvis, M.C., Duncan, H.J. 1989. *In vitro* digestibility of kale (brassica oleracea) secondary xylem and parenchyma cell wall and their polysaccharide components. *Journal of the Science of Foodand Agriculture, 48*, 9-14.

Wu, L., Xu, Y., Yang, Z., Feng, O. 2018. Hydroxytyrosol and olive leaf extract exert cardioprotective effects by inhibiting GRP78 and CHOP expression. *The Journal of Biomedical Research, 0*(0), 1–9

Yang, N., Jin, Y., Tian, Y., Jin, Z., & Xu, X. 2016. An experimental system for extraction of pectin from orange peel waste based on the o-core transformer structure*. Biosystems Engineering, 148*, 48–54.

Yener, M. E. (2015). Supercritical fluid processing for the recovery of bioactive compounds fromfood industry by-products. In T. Fornari, & R. P. Stateva (Eds.), High pressure fluid technology for green food processing (pp. 305–355). Springer International Publishing.

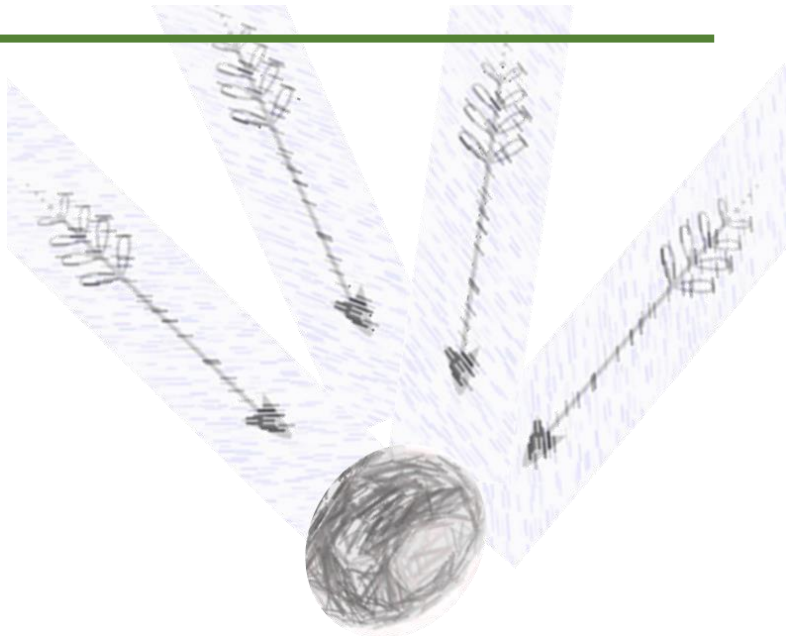
Zanetti, M., Carniela, T.K., Dalcantona, F.,… Fiori, M.A. 2018. Use of encapsulated natural compounds as antimicrobial additives in food packaging: A brief review. *Trends in Food Science & Technology, 81*, 51–60

Zhang, W.B., Xu, P., Zhang, H. 2015. Pectin in cancer therapy: A review. *Trends In Food Science & Technology, 44*, 258-271.

Zhang, X.; Cao, J.; Zhong, L.2009. Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX- 2 expression in human monocytic cells. *Naunyn*-Schmiedeberg's Archives of *Pharmacology*. 379, 581–586.

Zubair, H., Bhardwaj, A., Ahmad, A., Srivastava, S. K., Khan, M. A., Patel, G. K., et al. 2017. Hydroxytyrosol induces apoptosis and cell cycle arrest and suppresses multiple oncogenic signaling pathways in prostate cancer cells. *Nutrition and Cancer, 69*, 932–942.

4. Objetivos



4. Objetivos

La industria del aceite de oliva, consciente de la necesidad de eliminar los residuos medioambientalmente nocivos, está evolucionando para lograr el aprovechamiento integral de cada uno de los residuos que se generan de ella. El alperujo, subproducto mayoritario del proceso de extracción del aceite de oliva, retiene el 98% de los fenoles y otros compuestos minoritarios de las aceitunas. Está siendo tratado térmicamente para permitir la solubilización de una gran parte de compuestos de interés a la fracción líquida. Esta fracción líquida está siendo ya utilizada por la industria, y dos antioxidantes fenólicos muy activos, el hidroxitirosol (HT) y el 3,4-dihidroxifenilglicol (DHFG) están siendo recuperados. Esta tesis se centra en el estudio de estabilidad, biodisponibilidad y mejoras de la funcionalidad de estos dos compuestos fenólicos, así como en el estudio y recuperación de componentes de la pared celular con actividad biológica a partir de estas fracciones acuosas, entre los que destacan las pectinas. Precisamente han sido las propiedades de dichas pectinas, capacidad para actuar como vehículo de liberación de compuestos bioactivos y propiedades nutracéuticas y funcionales (aplicación en conservación de alimentos y en prevenir y/o combatir el cáncer), la que nos ha llevado a estudiarlas, ya sea comercial o aisladas del propio alperujo. Así, para desarrollar este trabajo de investigación se plantean los siguientes objetivos:

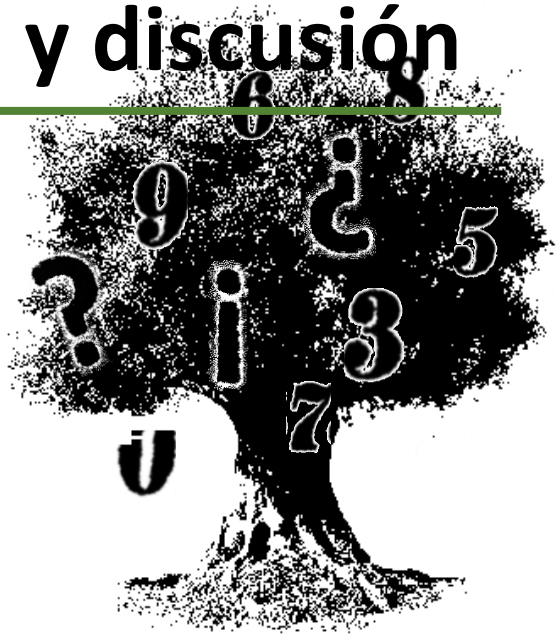
1.- Encapsular los dos antioxidantes, HT y DHFG, con reconocida eficacia preventiva y terapéutica en enfermedades intestinales inflamatorias mediante formulaciones de pectina (pectina amidada, pectina-alginato, pectina-alginato-aceite de oliva) con objeto que alcancen el intestino grueso y contribuya a la salud antioxidante intestinal.

2.- Formular recubrimientos y/o películas comestibles a base de pectina-gelatina de pescado y los antioxidantes HT y DHFG, con capacidad de proteger a los alimentos del ataque de microorganismos y de la degradación oxidativa.

3.- Obtener una fibra alimentaria (dietaria) funcionalizada con los fenoles antioxidantes HT y DHFG, que combine las propiedades funcionales de la fibra alimentaria y la de los antioxidantes, y que promuevan la salud intestinal.

4.- Evaluar el efecto en la proliferación *in vitro* sobre líneas celulares cancerosas de extractos ricos en pectinas modificadas con polifenoles asociados a partir de los subproductos de la extracción del aceite de oliva.

5. Resultados y discusión



BLOQUE I

Título: Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting[1].

Autores: Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Lama-Muñoz, & Juan Fernández-Bolaños

Publicación: Carbohydrate Polymers

Título: Molecular interactions between 3,4-dihydroxyphenylglycol and pectin and antioxidant capacity of this complex in vitro[2].

Autores: Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, Elisa Rodríguez-Juan, Alejandro González-Benjumea, & Juan Fernández-Bolaños

Publicación: Carbohydrate Polymers

Título: Anti-Inflammatory local effect of hydroxytyrosol combined with pectin-alginate and olive oil on trinitrobenzene sulfonic acid-induced colitis in wistar rats[3].

Autores: Ana Voltes, Alejandra Bermúdez- Oria, Guillermo. Rodríguez-Gutiérrez, Maria Luisa Reyes, Carolina Olano, Juan Fernández-Bolaños & Fernando de la Portilla

Publicación: Journal of Investigative Surgery

Resumen Bloque I

Las enfermedades inflamatorias intestinales consisten en procesos inflamatorios que afectan a el tubo digestivo de forma crónica. Entre estas enfermedades se encuentra la Colitis Ulcerosa y la Enfermedad de Crohn. Las molestias que conllevan estas enfermedades disminuyen notablemente la calidad de vida de los pacientes que las sufren. A día de hoy no existe una cura total, por lo que los tratamientos, terapias farmacológicas o cirugía, únicamente intentan controlar y reducir los síntomas.

Una dieta mediterránea se caracteriza en por un alto consumo en aceite de oliva, las evidencias ponen de manifiesto que el consumo de aceite de oliva podría presentar un fuerte efecto antinflamatorio. El aceite de oliva presenta un alto contenido en ácidos grasos monoinsaturados, así como componentes menores; eritrodiol, β-sitosterol, escualeno, tocoferoles, carotenoides y los compuestos fenólicos serían los responsables del efecto antinflamatorio. Debido a los numerosos compuestos antioxidantes presentes en el aceite de oliva y teniendo presente que en los procesos inflamatorios está implicado el estrés oxidativo, estos compuestos antioxidantes podrían presentar numerosos efectos beneficiosos en enfermedades inflamatorias como las intestinales. El hidroxitirosol (HT) es un fenol simple y se considera que es uno de los principales agentes antioxidantes presentes en el aceite de oliva con una potente actividad antiinflamatoria, además de anticancerígeno, antimicrobiano y antipirético. De hecho, la Autoridad Europea de Seguridad Alimentaria (EFSA) indica que si se consume ≥5 mg HT / día reduce el riesgo de padecer aterosclerosis. El 3,4-dihidroxifenilglicol (DHFG) es otro fenol simple presente en la fruta de olivo con la misma estructura orto-difenólica que el HT, pero con un grupo hidroxilo adicional en posición β. DHPG presenta una alta capacidad antioxidante y un elevado potencial antiinflamatorio.

Sin embargo, las propiedades farmacocinéticas del HT son desfavorables, ya que el compuesto muestra baja biodisponibilidad oral y rápida eliminación en humanos. Hasta la fecha no hay estudios de las propiedades farmacocinéticas del DHFG, pero debido a su similitud deberían ser similares.

En este trabajo se ha estudiado la interacción de la pectina con estos dos potentes antioxidantes presentes en la aceituna el HT y el DHFG. Debido a las propiedades farmacocinéticas que presentan los compuestos, se realizaron encapsulaciones de pectina amidada, pectina/alginato y pectina/alginato/aceite de oliva con HT o DHFG mediante el método de gelación iónica, con el objetivo de su liberación más lenta mejorando su efecto en el colón. Los resultados mostraron que después de una digestión gástrica e intestinal simulada, las encapsulaciones pueden retener los compuestos hidrosolubles HT y DHPG en cantidad suficiente para su efecto *in situ* en el colón y su posible beneficio en enfermedades inflamatorias intestinales. Debido a que esta cantidad encapsulada era prácticamente imposible recuperarla, se sugirió la formación de un complejo pectina-HT gracias a la purificación por cromatografía de exclusión de tamaño, cambios en el espectro de fluorescencia de HT-pectina respecto a un control sin HT, y el análisis MALDI TOF-TOF [1]. Mientras que para el estudio de la interacción DHFG-pectina los ensayos de actividad antioxidante exhibieron que el complejo formado seguía presentando actividad antioxidante después de la digestión simulada *in vitro*, lo que corroboraría la formación del complejo DHFG-pectina y su posible efecto *in situ* en el colón[2].

Así mismo, teniendo presente los efectos beneficiosos que el HT posee y la posible interacción HT-pectina, se han realizado ensayos *in vivo* en ratas Wistar a las cuales se les indujo una colitis en fase aguda mediante la administración de ácido trinitrobencenosulfónico (TNBS) con el objetivo de observar el efecto beneficioso in situ e *in vivo* del HT frente a enfermedades inflamatorias intestinales. En este estudio el HT fue emulsionado con aceite de oliva, pectina cítrica y alginato, el cual fue aplicado vía rectal a los animales de ensayo. Los resultados sugieren que el grupo de ratas tratadas con la emulsión que presentaba HT, disminuye la colitis ulcerosa, demostrando así su efecto antiinflamatorio y por tanto, su efecto en el tratamiento de la enfermedad de inflamación intestinal.

Esquema resumen bloque I.

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Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting

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ABSTRACT

Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) are two phenolic antioxidants naturally found in olive fruit with anti-inflammatory properties. This study explored the interaction of pectin with HT and DHPG via their encapsulation into pectinate beads. Purification by size exclusion chromatography, changes in the fluorescence spectrum of the HT and pectin, and MALDI TOF–TOF analysis suggested the existence of the phenol-pectin complexes. The entrapment efficiency, swelling properties, and *in vitro* release of HT and DHPG of the beads were studied.

The results show that the beads can entrap the water soluble compounds HT and DHPG in sufficient amounts to reach the colon. The beads consisted of an important amount of pectin-bound HT or DHPG after two hours at gastric pH. This study highlights the potential use of HT-and DHPG-loaded pectinate gel beads for the colon-targeted delivery of these bioactive compounds to help prevent or relieve chronic inflammatory bowel disease.

1. Introduction

Chronic inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's disease, two autoimmune disorders exacerbated by inflammatory mediators (Marks et al., 2006).

Evidence shows that virgin olive oil might exert beneficial effects on markers of inflammation (De la Puerta, Martínez-Domínguez, & Ruiz-Gutiérrez, 2000). The oil's high monounsaturated fatty acid content and the presence of minor components such as erythrodiol, β-sitosterol, squalene, tocopherols, carotenoids, and phenolic compounds, exert the anti-inflammatory effect (Lyons et al., 2016).

Considering the involvement of oxidative stress in inflammation, antioxidants might bring benefits in inflammatory diseases. In fact, virgin olive oil contains numerous antioxidant phenolic compounds that exert potent anti-inflammatory actions (Muto et al., 2015). In the last few years, several studies demonstrated that diets supplemented with olive oil and/or olive oil phenolics compounds exert a protective effect in experimental colitis in rodents, which may be mediated by their strongly anti-oxidative potential (Sánchez-Fidalgo et al., 2013; Takashima et al., 2014).

Hydroxytyrosol (HT) is a simple phenol and considered to be the main olive oil antioxidant with a potent anti-inflammatory activity (Bitler, Viale, Damaj, & Crea, 2005; Ciriminna, Meneguzzo, Fidalgo, Ilharco, & Pagliaro, 2016). The European Food Safety Authority (EFSA) allows the health claim that ≥5 mg HT/day prevents low-density lipoprotein (LDL) oxidation and, therefore, reduces the risk of atherosclerosis (EFSA NDA Panel, 2012). Thus, it is an indirect recognition of the anti-inflammatory effect of HT (Pontoniere & Martiradonna, 2012). 3,4-dihydroxyphenylglycol (DHPG) is another simple phenol present in the olive fruit with the same *ortho*-diphenolic structure as HT but with an additional hydroxyl group in the β position. DHPG also has powerful antioxidant properties and potentially has anti-inflammatory properties (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007).

In this paper, we propose the delivery of HT, DHPG, or olive oil to the colon as agents capable of preventing or improving IBD. The encapsulation of HT or DHPG with calcium-pectinate beads as a colon-targeted delivery system will be assessed for the first time. Moreover, beads will be prepared by the emulsion method using olive oil to form an oil-in water emulsion with citrus pectin act-

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ing as the emulsifier. Pectin is naturally present in plant cell walls and possesses an interesting potential for the delivery of drugs to the colon (Das & Ng, 2010). It is specifically biodegraded by colonic bacteria and has been found to inhibit both local and systemic inflammation and prevent intestinal inflammation (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Popov et al., 2013). To our knowledge, there has been no study to date on the encapsulation of HT, combined with olive oil and pectin – all of which have anti-inflammatory properties – for the local treatment of bowel diseases.

The preventive and therapeutic efficacy of HT for IBD is well documented (Sánchez-Fidalgo, Sánchez de Ibarguen, Cardeno, & Alarcón de la Lastra, 2012; Takashima et al., 2014). However, due to its rapid absorption and metabolism – reaching the maxima plasma concentration after oral administration in 5–10 min followed by rapid decline (D'Angelo et al., 2001) and with a very short elimination half-life of 2.43 h (Miró-Casas et al., 2003) – an insufficient amount of HT will reach the colonic region.

2. Hypotheses

Our hypothesis for the current study was a delayed release of HT or DHPG with the delivery system in the colonic region, thus providing a novel possibility for the treatment of oxidative-stress mediated inflammatory disease.

The objective of the present investigation was to compare the entrapment efficiency and release profiles of HT and DHPG using three different types of beads based on the natural polysaccharides pectin and alginate in combination with and without virgin olive oil and amidated pectin. The beads were treated *in vitro* through dissolution conditions that mimic the gastric to colonic transit. The interactions between pectin and HT were also studied in order to check their efficacy for colon targeting.

3. Material and methods

3.1. Materials

HT and DHPG were extracted and purified from olive by-products using a chromatographic system following the processes described by Fernández-Bolaños et al. (2011, 2014). Citrus pectin with a high degree of esterification (53%), and sodium alginate were purchased from Sigma-Aldrich (St Louis, MO, USA). Amidated pectin, with a low degree of esterification (30%) and a 21% degree of amidation was a generous gift by Herbstreith & Fox KG (Neuenbürg, Germany). Virgin olive oil was purchased from a local supermarket. Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) and a mixture of pectinolytic enzymes from Novozyme Corp. were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Preparation of formulations

Calcium pectin-alginate beads and amidated pectin beads were instantaneously produced by the ionotropic gelation method. Citrus pectin (0.8 g) and sodium alginate (0.2 g), or 1.0 g of amidated pectin were homogeneously dispersed in 30 mL of aqueous solution containing HT (65%) or DHPG (95%) using a homogenizer (Ultra-Turrax ® T50 Basic, IKA, Germany) at a speed of 24,000 rpm for 5 min in an ice-bath to avoid overheating. The pectin dispersions containing HT or DHPG were dropped into 30 mL of 10% w/v $CaCl_2$ solution using a nozzle with an inner diameter of 1.2 mm. The beads formed were allowed to stand in the $CaCl_2$ solution for different cross-linking times (from 2 to 30 min) until an optimized curing time. HT/DHPG loaded beads gels were separated from the $CaCl_2$ solution by filtration and washed with distilled water. In the filtrated and washed liquid were measured the free HT/DHPG that was not entrapped within the beads. The separated gel beads were dried at 37 °C for 48 h in an air-circulated oven until a constant weight was obtained.

Pectin-alginate beads containing olive oil were prepared by the emulsion-gelation method. 7.5 g of virgin olive oil was added to the polymers and HT/DHPG solution. The mixture was emulsified using the same homogenization conditions and the olive oil-incorporated into emulsion gel beads were treated in the same manner as the calcium pectin-alginate beads.

3.3. Extraction and analysis of HT and DHPG from beads and emulsions

Extraction of HT/DHPG from the hydrocolloids (beads and emulsions) was evaluated in aqueous solutions and various organic solvents. The unbound phenolics were extracted as follows: 0.1 g of dry beads or emulsion was dispersed in 100 mL of acidified water for 2 h at 37 °C in a shaking water bath. The quantities of HT or DHPG were determined by HPLC according to a previously published method (Rodríguez et al., 2007). The extraction of strongly bound HT/DHPG was also assayed according to the method of Nordkvist, Salomonsson, & Åman, (1984), using 1 M NaOH containing 0.5% sodium borohydride for 20 min. The hydrocolloids were then subjected to acid hydrolysis according to the method of Graciani & Vázquez (1980). 0.1 g of dry beads/emulsion were treated with 10 mL of 3 N HCl in a homogenizer Ultra-Turrax (24,000 rpm for 5 min) and then heated to 100 °C for 10 min and filtered.

Also 0.1 g of the beads or emulsion were extracted with 25 mL of organic solvent by stirring (30 min), sonication (15 min), and Ultra-Turrax (24,000 rpm for 5 min), using methanol:water (20, 40, 80%) and dimethyl sulfoxide (DMSO): water (10, 30, 60, 90%). All the dispersions were filtered. In any case it was possible to quantify the strongly bound HT or DHPG. Also, due to the difficulty of dissolving the dry beads, the HT/DHPG loaded dried hydrogel beads were broken down by immersion in sodium phosphate buffer (50 mM, pH 6.8) containing 5 mM of EDTA (Ethylenediaminetetraacetic acid). A preliminary acid treatment was necessary to help the erosion of the matrix (Nguyen, Winckler, Loison, & Wache, 2014): Beads (0.1 g) were dispersed in 100 mL of 0.1 M HCl for 2 h at 37 °C in a shaking water bath. After filtration, the beads were further treated by sodium phosphate buffer with EDTA at 37 °C until dissolution. An alkali extraction ($NaHCO_3$, pH 10) at 100 °C for 30 min was also tested.

3.4. Entrapment efficiency (EE) and bioactive compounds loading (BCL)

The EE of the bioactive compounds (HT/DHPG) was calculated indirectly by determination of the free HT/DHPG content in the aqueous solution after filtration and washes, according to the following equation: EE (%) = [(Qt-Qr)/Qt] x 100, where Qt is the bioactives content initially added during the bead loading, and Qr is the sum of content recovered in the aqueous solution after separating and washing the beads.

The amount of bioactive compounds (HT/DHPG) present in the beads (bioactive compounds loading, BCL) was also determined by an indirect method according to the following equation: BCL (%) = [(Qt-Qr)/Wp] x 100, where Qt is the bioactives content initially added during the bead loading, Qr is the sum of content recovered in the aqueous solution after separating and washing the beads, and Wp is the total weight of dry beads recovered per batch. All experiments were performed in triplicate.

3.5. Swelling studies

Samples of HT-loaded beads from the three preparations were taken, weighed, and placed in a beaker containing 100 mL of 0.1 M HCl (pH 1.2) maintained at 37 °C. The beads were periodically removed at predetermined intervals and weighed. The swelling ratio was calculated as the weight of wet beads/weight of dried beads.

3.6. . In vitro *release studies*

The HT/DHPG loaded dried hydrogel beads (0.1 g) were immersed in 100 mL at pH 1.2 with 0.1 M HCl solution (simulated gastric juice) at 37 °C with gentle shaking for the first 2 h, and then in a pH 6.8 sodium phosphate buffer solution (simulated intestinal juice) for the following 2–3 h. At given time intervals, 0.1 mL samples were withdrawn, replaced with an equal volume of fresh medium, and assayed for HT content by HPLC. The amount of HT/DHPG released was plotted versus time (mean of two determinations, coefficient of variation CV < 1.5%).

3.7. Stability of the beads binding of HT/DHPG with pectin during storage

The beads batches were stored for 30 or 60 days, respectively, in four different conditions: cold (4 °C), room temperature (RT), 37 °C, and 50 °C, in order to investigate the possible effect of different storage conditions on the binding of phenols-pectin or the stability of the phenols. The samples were stored in triplicate for each time point. The samples were analyzed for HT/DHPG content at predetermined time intervals (1–3, 15, 30, 50, and 60 days) by HPLC using the extraction procedures described in epigraph 2.3 using an extraction with methanol:water (40:60).

3.8. Purification of hydroxytyrosol-pectin complex

Size exclusion chromatography on two-column Superdex Peptide HR 10/30 (30 × 1 cm) (Pharmacia Biotech, Uppsala, Sweden) connected in line to a Jasco LC-Net II/ADC HPLC (Easton, MD, USA) was used to purify the HT-pectin complex. 1.0 g of samples of dried HT-loaded pectin-alginate beads and amidated pectin beads containing 87.5 mg and 138.5 mg of HT, respectively, were dissolved in 100 mL of phosphate buffer (50 mM, pH 6.8) containing 5 mM EDTA and a mixture of pectinolytic enzymes (4 μg/mL) – including *endo*- and *exo*-polygalacturonase (PG) and pectinesterase (PE) (Novo Nordisk, Bagsvaerd, Denmark) – incubated at 37 °C for 24 h, applied (100 μL) four times onto the column, and eluted with distilled water at a flow rate of 0.5 mL/min. The peaks were monitored with a Jasco FP-2020Plus Fluorescence detector and a Shodex RI-71 Refraction Index detector. Column calibration was performed with galacturonic acid and tri-galacturonic acid (Fluka) as standards for the pectic oligosaccharides. The beads were previously treated with 100 mL of 0.1 M HCl (pH 1.2) at 37 °C for 2 h to remove all the unbound HT and to help break down the beads. Samples of hydrogel beads (pectin-alginate and amidated pectin) not loaded with phenol were used as control and treated in a similar manner.

A series of fractions shorter than that of the original pectin collected by the Superdex Peptide column were also separated by HPLC using a Kinetex EVO C18 analytical column (250 × 4.6 mm i.d.; particle size 5.0 μm) (Phenomenex, USA). To identify differences between the samples with HT and the control in the chromatogram with fluorescence detection, a Jasco LC-Net II/ADC HPLC (Easton, MD, USA) system with a fluorescence detector (Jasco FP-2020Plus) was used. The mobile phases were 0.01% tricholoracetic acid in water and acetonitrile, with the following gradient during a total run time of 55 min: 95% A initially, 75% at 30 min, 50% at 45 min, 0% at 47 min, 75% at 50 min, and 95% at 52% min until the run was complete.

The HT-loaded and control beads were analyzed by NMR spectroscopy. ^{1}H NMR spectra were recorded at 500.1 MHz on a Bruker Avance-500 spectrometer using D_2O as solvent. Chemical shifts were reported in δ units (ppm) values relative to the central solvent peak D_2O set at 4.79.

MALDI-TOF mass analysis of the isolated fractions of Superdex Peptide HR was performed using an UltrafleXtreme Bruker mass spectrometer Smartbeam-II laser. The MALDI-TOF mass spectra were acquired in the negative and positive ion mode over a mass-to-charge ratio (m/z) range of 700–3500 Da. The instrument was operated at an accelerating voltage of 26.45 kV with an extra voltage of 13.399 kV. Each spectrum was produced by accumulating data from 1000 to 2000 laser shots. The matrix solution of HCCA (alpha-cyano-4-hydroxycinnamic acid) was prepared in 10 mg/mL ACN:H_2O:TFA (50:47.5:2.5) (v/v/v) in the presence of sodium trifluoroacetate. The samples were mixed with the matrix solution in a v/v ratio of 1:100.

4. Results and discussion

4.1. Preparation of hydroxytyrosol (HT)/3,4-dihydroxyphenlyglycol (DHPG)–loaded gel beads. Entrapment efficiency and entrapment loading

The beads were prepared by emulsion-gelation method using virgin olive oil to form an oil-water emulsion, with citrus pectin acting as an emulsifier. The citrus pectin with a high degree of esterification (DE) used is unable to cause the gelation process in the presence of calcium ions, but it is a natural surface-active agent that reduces the interfacial tension between oil and water phases (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, García, & Fernández-Bolaños, 2015). In combination with sodium alginate, with a pectin-alginate ratio of 80:20, the use of calcium as a counter-ion agent led to a high degree of alginate chain crosslinking during gelation. Also, calcium pectin-alginate beads without olive oil, and calcium-amidated pectinate were assessed for HT/DHPG loading. When an amide group is introduced in low DE pectin, the hydrophilic property is reduced and there is an increasing tendency to form a gel in the presence of calcium (Berg, Bretz, Hubbermann, & Schwarz, 2012).

HT and DHPG, two phenolic antioxidant molecules naturally found in olive fruit, have a high solubility in water and a good solubility in oil too (higher in the case of HT). In the present study, HT with 65% purity and a DHPG with 95% purity were used. The polymeric beads of pectin-alginate and amidated pectin revealed similar entrapment efficiencies (EE) during formulation for DHPG and for increasing amounts of HT, about 53% EE (Table 1). Only the olive oil entrapped in the pectin-alginate formulation acted as a certain barrier and the EE was slightly lower at 46%. These interesting EE values were obtained with a curing time of 2 min in the calcium solution. With an increased curing time of 30 min, the EE was reduced to just 20–30% in all preparations and for all concentration of HT. This fact may be explained by the high water solubility of HT and DHPG and their diffusion out of the matrix into the $CaCl_2$ solution during bead formation.

The entrapment or bioactive compounds loading (BCL) in mg/100 mg of dry beads is presented in Table 1. The loading of beads with HT increased when the initial HT amount in the pectin-alginate or amidated pectin solution was increased. The highest HT content was obtained by preparing the beads with amidated pectin and an initial amount of 750 mg HT, in which case the HT load varied between 12.9% ± 0.98 mg/g dry beads and 13.9% ± 0.04, for the pectin-alginate without olive oil and amidated pectin, respectively.

Table 1
Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) content of beads: entrapment efficiency (EE) and bioactive compounds loading (BCL). Retention of bioactive compound (BC) in simulated gastric fluid (pH 1.2, 0.1 M HCl solution) for 2 h of hydrolytic process and binding capacity (mg BC/g dry beads).

Types of beads	BC (mg)		EE (%) ± SD	BCL (%) ± SD	Retention (%) ± SD	Binding capacity (mg/g dry beads)
Pectin-Alginate-Virgin olive oil	HT	200	47.26 ± 2.80	0.94 ± 0.04	26.87 ± 4.87	2.53
		500	46.64 ± 0.51	2.28 ± 0.01	31.88 ± 2.78	7.27
		750	46.52 ± 1.49	3.28 ± 0.01	18.59 ± 1.22	6.10
		1000	45.87 ± 1.53	3.39 ± 0.15	22.85 ± 0.86	7.75
	DHPG	100	47.90 ± 0.09	0.48 ± 0.01	69.92 ± 2.29	3.35
Pectin-Alginate	HT	200	53.64 ± 0.19	4.34 ± 0.34	22.89 ± 0.75	9.93
		500	55.76 ± 3.82	9.81 ± 1.32	26.11 ± 4.53	25.61
		750	55.50 ± 4.63	12.87 ± 0.98	23.44 ± 1.40	30.17
		1000	51.48 ± 3.12	10.30 ± 0.63	35,50 ± 0.75	36.57
	DHPG	100	49.70 ± 0.73	3.45 ± 0.14	67.41 ± 1.92	23.25
Amidated Pectin	HT	200	52.99 ± 1.13	4.82 ± 0.05	22.77 ± 2.80	10.98
		500	53.32 ± 1.13	11.60 ± 0.01	20.34 ± 4.72	23.60
		750	52.88 ± 1.42	13.90 ± 0.04	22.79 ± 4,34	31.68
		1000	46.30 ± 0.97	13.86 ± 0.20	36.00 ± 1.98	49.86
	DHPG	100	57.29 ± 1.39	2.53 ± 0.17	69.72 ± 1.52	17.64

However, the loading efficacy was reduced when the beads were prepared with olive oil. In this case, the amount of olive oil added to the preparation should be taken into account for the increased dry weight of the beads. These results were subsequently used in the dissolution studies to calculate the percentage of HT/DHPG retention.

4.2. In vitro *studies of HT/DHPG release from gel beads*

The *in vitro* dissolution studies observed the release rate of encapsulated HT/DHPG from the beads and HT/DHPG retention in simulated gastric fluid (pH 1.2, 0.1 M HCl solution, 2 h) (Table 1).

The HT retention results showed similar values for all bead preparations, between 19 and 36%, whereas the retention of DHPG was higher, reaching 70%. However, the binding capacity (in mg of HT retained per g of dry beads) was higher for amidated pectin (50 mg of HT/g dry beads) than for pectin-alginate preparation (37 mg of HT/g dry beads) for an initial load of 1000 mg of HT. In the case of pectin-alginate containing olive oil, the HT binding capacity was lower, with a maximum of 8 mg/g dry beads, due to the increase in the dry weight of the beads due to the olive oil added. In the case of DHPG, due to the high retention and only 100 mg of initial load, higher binding capacity values were obtained for amidated (17.6 mg of DHPG/g dry beads), pectin-alginate (14.3 mg of DHPG/g dry beads), and pectin-alginate-virgin olive oil (3.3 mg of DHPG/g dry beads) compared to those obtained for an initial HT load of 200 mg (11.0, 9.9, and 2.5 mg of HT/g dry beads, respectively).

The course of HT and DHPG release was similar for all the hydrogel beads. Within the first 10 min, there was a strong release of HT followed by a slower continuous release. The release of DHPG was slower for both amidated pectin and for pectin-alginate containing olive oil preparation (Fig. 1). However, following the pH change simulating intestinal fluid (pH 6.8, phosphate buffer) at the 2 h timepoint, only 1–2% of HT/DHPG was released in all formulations. All the beads were still apparently intact after 2 h immersion in the simulated gastric environment. The phosphate buffer induces the disintegration of the beads due to the capture of calcium ions by phosphate ions in the medium (Dhalleine et al., 2011), and consequently, the total release of HT/DHPG was expected. However, phosphate-induced bead disintegration did not provide the total release of HT or DHPG, a phenomenon that can be explained by the interaction between HT/DHPG and pectin (both amidated and non amidated). It was also verified that both HT and DHPG remain totally stable in simulate gastric and intestinal environment.

Subsequent release studies carried out in the presence of pectinolytic enzymes, and even with the content of rat caecum, did not show the expected liberation of HT as a consequence of the selective hydrolysis and fermentation of the pectin-alginate beads. Different swelling ratios were also observed for the different formulations of HT-loaded beads (Fig. 2). The presence of olive oil in the pectin-alginate formulation acted as a barrier, as the percentage of swelling was approximately 8-fold lower than for beads of the same formulation without olive oil. These results suggest that the dried beads and the HT-loaded pectin-alginate beads in particular, will swell slightly in the stomach and the HT would undergo a quite rapid release. Subsequently, the beads would be transferred to the upper intestine and behave as a matrix for the controlled release of the remaining HT. However, the interaction of HT with the pectinate matrix was strong and a large fraction of the encapsulated HT would remain strongly bound to the pectin.

4.3. *Investigation of the interaction between HT and pectin*

The breakdown of the interaction between HT and pectin was assessed in different alkaline and acid hydrolysis conditions and by extraction with different organic solvents. The alkali degradation of HT and the extraction with HCl were similar or slightly less efficient than extraction with MeOH (data not shown); therefore, extraction with MeOH was chosen. This result suggests that it is only possible to quantify the unbound HT and that the strong complex formed between HT and pectin is not quantifiable.

The interaction between HT and pectin and the influence of drying temperature and/or storage conditions on their formation was investigated for calcium pectin-alginate beads. The binding percentage was estimated from HT release data. As shown in Fig. 3, the formation of the beads was notably affected by the drying temperature and storage conditions. From day three, a retention of about 30–35% occurred, even at 4 °C. Further, after 50 and 60 days of storage at RT, 37 °C, or 50 °C, the percentage remaining in the beads was notably increased from 44 to 70%.

Some interactions between polyphenols and certain classes of polysaccharides have been reported (Renard, Baron, Guyot, & Drileau, 2001). Specifically, the intermolecular interaction between pectin and tannin (a high molecular weight polyphenol) has been described (Taira, Ono, & Matsumoto, 1997). Moreover, the persimmon astringency of gallate-type catechin was reduced by

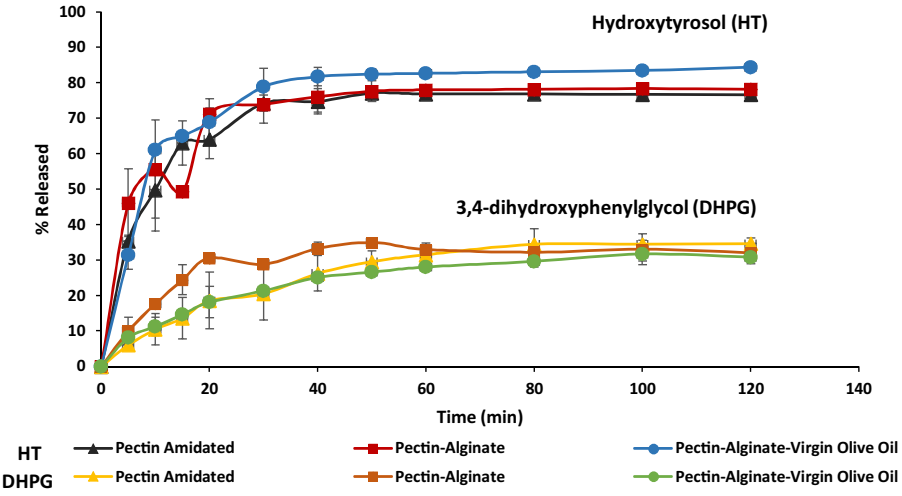

Fig. 1. *In vitro* release profiles of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from Ca-pectin-alginate beads with or without virgin olive oil and amidated pectin after incubation in simulated gastric fluid. Mean values (n = 3) are presented.

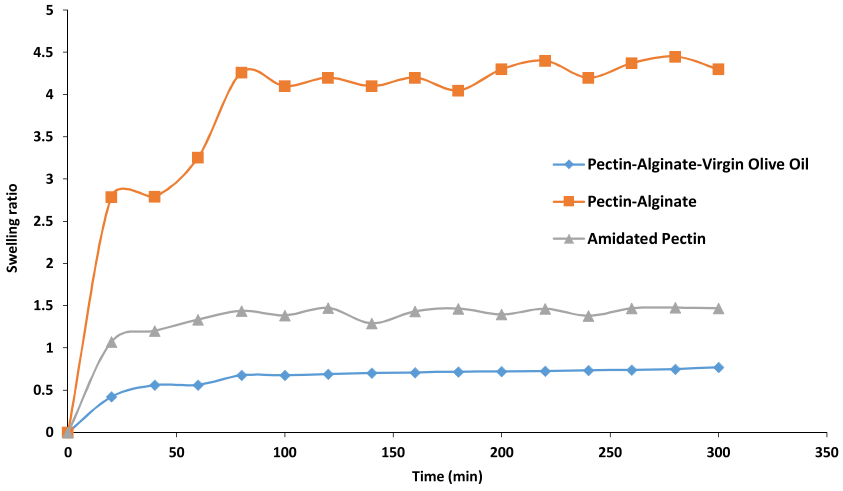

Fig. 2. The swelling ratio of the three formulations of hydroxytyrosol-loaded beads in 0.1 N HCl against time.

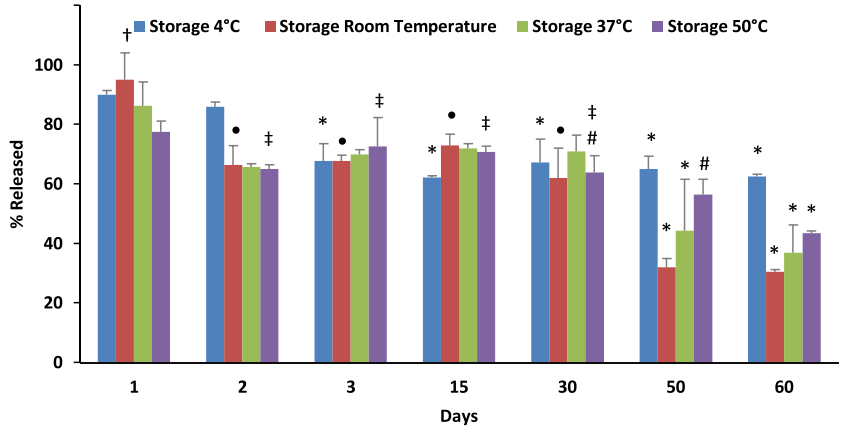

Fig. 3. Released percentage of hydroxytyrosol (HT) in HT- loaded –Ca-pectin-alginate beads at different storage conditions. The bars on the data represent standard deviations (n = 3). Storage at 4 °C: $^{*}p < 0.05$ for the difference between 3, 15, 30, 50, and 60 days compared with 1 and 2 days. Storage at RT: $^{*}p < 0.05$ for the difference between 1–3, 15, and 30 days compared with 50 and 60 days; $^{\ddagger}p < 0.05$ for the difference between 2, 3, 15, and 30 days compared with 1, 50, and 60 days; $^{\dagger}p < 0.05$ for the difference between 1 day compared with 2, 3, 15, 30, 50, and 60 days. Storage at 37 °C: $^{*}p < 0.05$ for the difference between 1–3, 15, and 30 days compared with 50 and 60 days. Storage at 50 °C: $^{*}p < 0.05$ for the difference between 1–3, 15, 30, and 50 days compared with 60 days; $^{\#}p < 0.05$ for the difference between 1–3, 15, and 60 days compared with 30 and 50 days; $^{\ddagger}p < 0.05$ for the difference between 2, 3, 15, and 30 days compared with 1, 50, and 60 days.

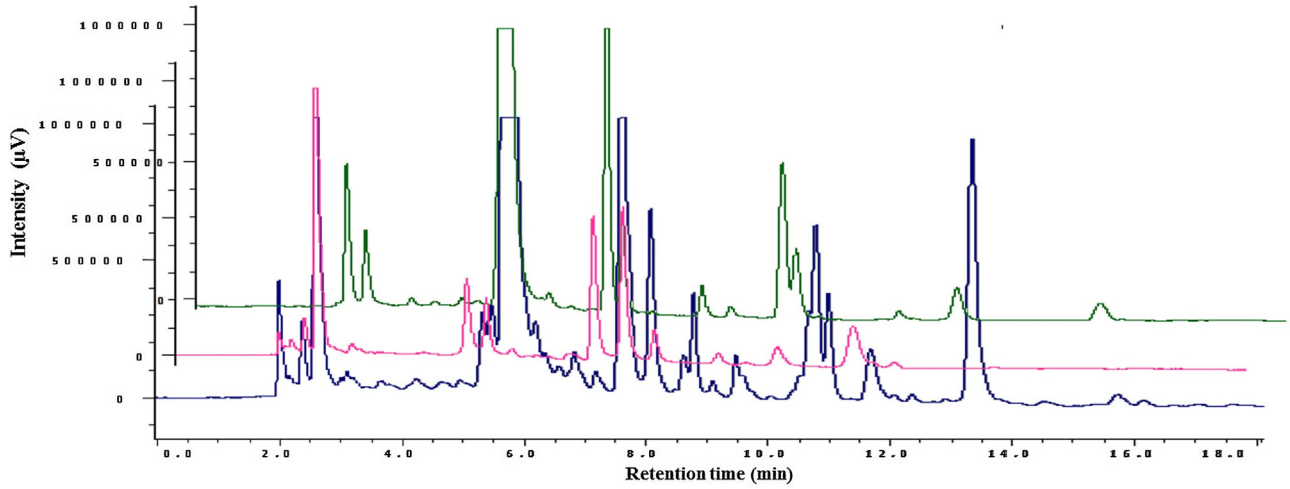

Fig. 4. HPLC chromatograms on reverse phase column and fluorescence spectroscopy detector of hydroxytyrosol alone (green), amidated pectin alone (pink) and hydroxytyrosol-amidated pectin mixture (blue) from beads disintegrated in pH 6.8 phosphate buffer plus 5 mM EDTA in combination with pectin degrading enzymes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

complexation with pectin (Hayashi, Ujihara, & Kohata, 2005), and pectin was reported to stabilize anthocyanins during processing by ionic interaction between the anthocyanin cation and the carboxylic function of the pectin backbone (Maier, Fromm, Schieber, Kammerer, & Carle, 2009).

Based on the chemical structure of HT, with an hydroxyl group in *ortho* position and an additional alcoholic hydroxyl group, the high affinity of HT for pectin could be attributable to a complex association or non-covalent association, which would not be easily detectable. We have demonstrated that this complex was not hydrolyzable, not detectable by UV absorption, and not detectable by high resolution ^{1}H NMR (data not shown). This last result is in agreement with the findings that the complexation of polyphenols with certain classes of polymers, including pectin, was not detectable by ^{1}H NMR (Hayashi et al., 2005; Velikov, 2009).

To provide more information about the HT-pectin interaction, an experiment with two different types of beads with a high HT content, calcium pectin-alginate (87.5 mg HT/g dry bead) and calcium amidated pectin (138.5 mg HT/g dry bead), and the corresponding control beads without HT was carried out. The beads were immersed in simulated gastric fluid at 37 °C for 2 h, filtrated and washed to remove the HT unbound, and then immersed in simulated intestinal fluid to completely disintegrate the beads. Finally, a mixture of pectinolytic enzymes was added to reduce the degree of polymerization. Certain differences between the samples containing HT and the control without HT were appreciated by separation by HPLC on reverse phase column and fluorescence spectroscopy detection. Several novel peaks, such as the intense peak at 13.5 min and the double peak at 8.7 min for HT-pectin, and certain retention times were shifted in the chromatogram (Fig. 4). For example, the unbound HT peak at 3.0 min was shifted to the earlier time of 2.0 while the peak at 10.5 min was shifted to the later time of 11 min for HT-loaded amidated pectin beads.

In addition, size exclusion chromatography fractionation of HT-pectin complex by Superdex Peptide HR10/30 was used to gain a better understanding of the assumed HT-pectin interaction with a molecular weight distribution followed by refractive index detection (Fig. 5). Treatment of the HT-loaded amidated pectin with pectinolytic enzymes resulted in the destruction of the peak at 30 min.

A series of fractions shorter than that of the original pectin were also selected and separated by HPLC. Differences were found in the chromatogram with fluorescence detection between the samples of HT-loaded amidated pectin and the control without HT (Fig. 6). Fractions show several shifted peaks (for example fractions II at 5.5 min) and new peaks (for example fraction IV at 8.3 min). Similar results were obtained from calcium pectin-alginate beads after Superdex Peptide HR 10/30 purification, with the changes in the fluorescence spectrum in the samples with HT.

Fraction II obtained from amidated pectin and eluted at a retention time of 45–55 min in size exclusion molecular chromatography was further analyzed, next to standard oligosaccharide with DP 3 according to reference compounds, by the MALDI-TOF TOF mass technique. In positive mode, a signal with mass *m*/*z* 1078 was observed that was not present in the corresponding control without HT (Fig. 7). After studying all the possible combinations of different oligosaccharides with galacturonic acid units, including with amide or methyl-ester groups, and the corresponding possible adducts of sodium, we were unable to compose a structure with this *m*/*z* value. A tentative structure is proposed for the formation of a complex or cluster between an oligosaccharide composed of three amidated galacturonic acid- and two methyl esterified galacturonic acid-type build units with a fragment ion [M+H^{+}] at *m*/*z* 924 and a molecule of HT (154 Da) that gives the mass *m*/*z* of 1078 (Fig. 7). The ion at *m*/*z* 877, with a difference of 201 *m*/*z* units with respect to the molecular ion of the complex, may be attributed to the loss of HT (−154 Da), and —OCH_3 (−31 Da) and —NH_2 (−16 Da) moieties. The mass differences between the signals at *m*/*z* 861 and 845 (16 *m*/*z* units) represent the loss of the other two amidated groups.

Non-covalent interactions, such as H-bonds, were also suggested for the association between polyphenol and cell wall material (Renard et al., 2001), specifically between pectin and gallic, caffeic, or coumaric acids in a model system (Tsai, Sun and Hsiao, 2010). However, to our knowledge, this current study is the first to propose and identify a complex between pectin and an *ortho*-phenol such as HT by MALDI-TOF TOF mass spectroscopy.

Our results suggest that HT forms a very strong complex with pectin, attributable to many sites of hydrogen bonding between the pectin hydroxyl groups and the HT phenolic OH groups that might be in the form of loose helices (Renard et al., 2001). This would explain the higher binding percentage of DHPG with pectin, with 70% retention in simulated gastric fluid after 2 h of hydrolytic process. DHPG is another simple phenol, structurally similar to HT

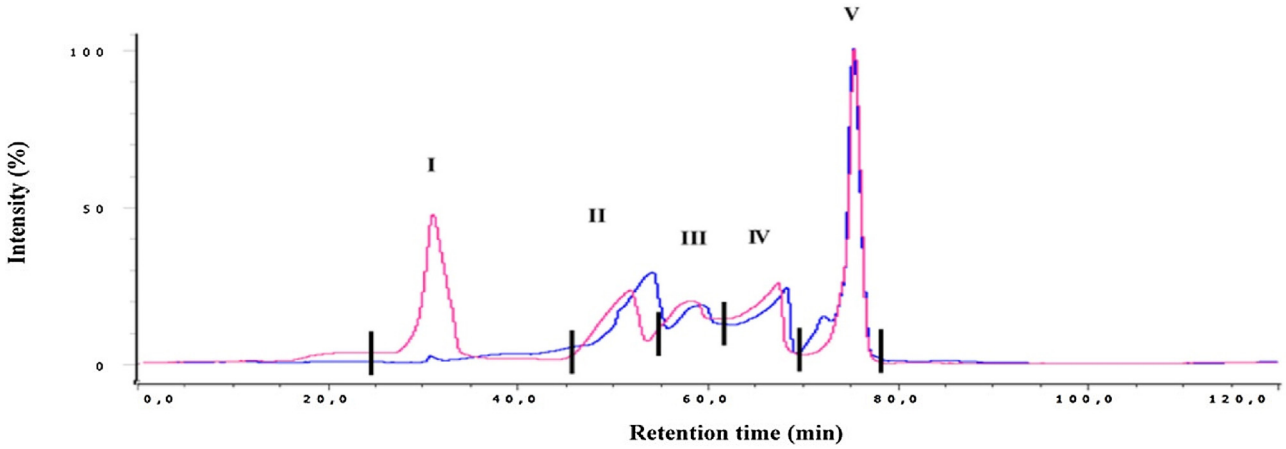

Fig. 5. Refractive index elution profiles by size exclusion chromatography using a Superdex Peptide column of the samples of the hydroxytyrosol-loaded amidated pectin treated with pectin degrading enzymes (blue) and comparison with non-enzyme treated samples (pink). The standard solutions of tri- and monogalacturonic acid had retention times of 52 and 69 min, respectively. In fraction V the main peak corresponds with solvent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

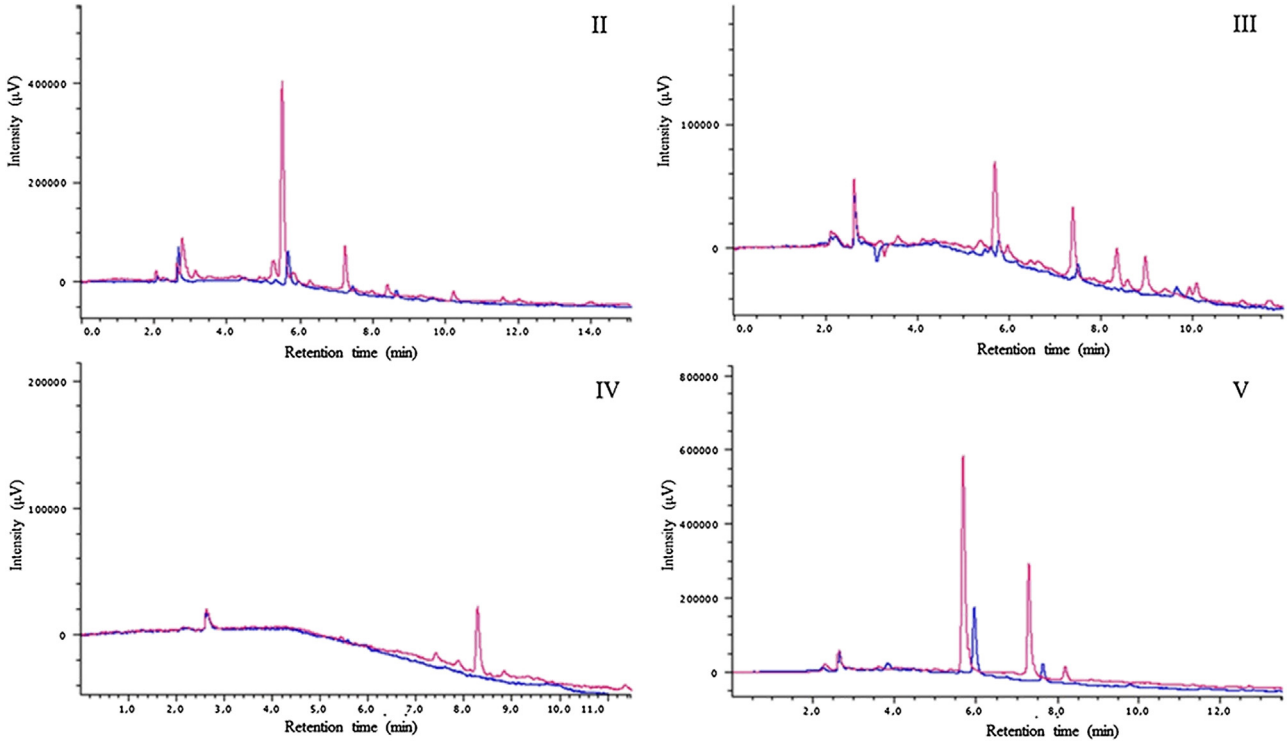

Fig. 6. HPLC chromatograms on reverse phase column and fluorescence spectroscopy detector of four fractions labeled II–V and collected by Superdex Peptide column from the sample of amidated pectin beads containing HT (pink) and the control without HT (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but with an additional hydroxyl group that contributes to more H-bond formation.

5. Conclusions

The present study shows for the first time that two highly water soluble polyphenols present in olive oil, HT and DHPG, can be encapsulated into a pectin-based formulation in high amounts (around 50%). The hydrogel beads showed a particular release pattern of the bioactive compounds in spite of the apparently intact appearance of the beads after 2 h immersion in the simulated gastric environment. For the three types of beads examined an important amount of HT (20–30%) and DHPG (70–80%) immobilized by interactions with the pectin polymer that arrive intact to the colon. To the best of our knowledge, the results reported here present the first evidence of the complexation of these phenolic compounds with pectin. This HT-pectin complex was not detectable by high resolution ^{1}H NMR but after purification we suggest its presence by MALDI TOF–TOF analysis.

In this paper we propose a novel potential delivery system for the delayed release of the HT/DHPG-pectin complex into pectinate beads for colon targeting. This last promising application, together with the anti-inflammatory effect of virgin olive oil (Muto et al., 2015) and citrus pectin (Popov et al., 2013), would be an interesting system for oral administration to prevent or improve IBD *in situ*. Further studies are required to evaluate the antioxidant or anti-

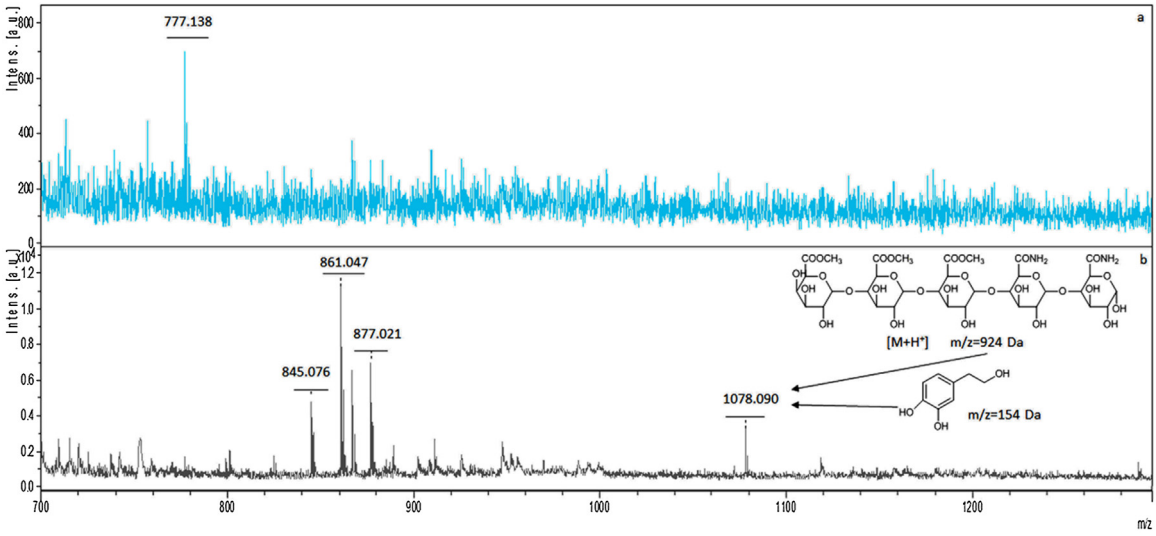

Fig. 7. MALDI-TOF TOF mass spectra (positive mode) of fraction II obtained from amidated pectin treated with pectinolytic enzymes and collected by Superdex Peptide column. a) Sample control without HT b) Sample with the complex HT-pectin.The signal intensity is plotted against the mass to charge ratio *m*/*z*. The proposed structure of a complex between a pentamer of amidated pectin and hydroxytyrosol gives the mass *m*/*z* of 1078.

inflammatory properties of the HT-pectin complex *in vivo*. We are currently aiming to address these requirements.

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References

Berg, S., Bretz, M., Hubbermann, E. M., & Schwarz, K. (2012). Influence of different pectins on powder characteristics of microencapsulated anthocyanins and their impact on drug retention of shellac coated granulate. *Journal of Food Engineering*, *108*, 158–165.

Bitler, C. M., Viale, T. M., Damaj, B., & Crea, R. (2005). Hydrolyzed olive vegetation water in mice has anti-inflammatory activity. *The Journal of Nutrition*, *135*, 1475–1479.

Ciriminna, R., Meneguzzo, F., Fidalgo, A., Ilharco, L. M., & Pagliaro, M. (2016). Extraction, benefits and valorization of olive polyphenols. *European Journal of Lipid Science and Technology*, *118*, 503–511.

D'Angelo, S., Manna, C., Migliardi, V., Mazzoni, O., Morrica, P., Capasso, G., et al. (2001). Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metabolism and Disposition*, *29*, 1492–1498.

Das, S., & Ng, K. Y. (2010). Colon-specify of reveratrol: Optimization of multi-particulate calcium-pectinate carrier. *International Journal of Pharmaceutics*, *385*, 20–28.

De la Puerta, R., Martínez-Domínguez, E., & Ruiz-Gutiérrez, V. (2000). Effect of minor components of virgin olive oil on topical antiinflammatory assays. *Zeitschrift Fur Naturforschung*, *55*, 814–819.

Dhalleine, C., Assifaoui, A., Moulari, B., Pellequer, Y., Cayot, P., & Lamprecht, O. (2011). Zinc-pectinate beads as an *in vivo* self-assembling system for pulsatile drug delivery. *International Journal Pharmaceutical*, *414*, 28–34.

EFSA NDA Panel. (2012). Scientific Opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal*, *10*, 2848. http://dx.doi.org/10.2903/j.efsa.2012.2848

Fernández-Bolaños, J., Guillén, R., Jiménez, A., Rodríguez, R., Rodríguez-Gutiérrez, G., & Lama-Muñoz, A., (2011). Method for purifying 3,4-Dihydroxyphenylglycol (DHPG) from plant products. International publication number WO 2010/070168.

Fernández-Bolaños Guzmán, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Rubio-Senent, F., Fernández-Bolaños Guzmán, J. M., Maya, I., López, O., & Marset, A., (2014). Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroyphenylglycol extract, and hydroxytyrosol acetate extract, from by-products of the olive tree, and the purification thereof. International publication number WO 2013/007850.

Graciani, E., & Vázquez, A. (1980). Estudio de los componentes del aceite de oliva por cromatografía líquida de alta eficacia (HPLC). II Cromatografía en fase inversa. *Grasas Y Aceites*, *31*, 237–243.

Hayashi, N., Ujihara, T., & Kohata, K. (2005). Reduction of catechin astrigency by the complexation of gallate-type catechins with pectin. *Bioscience Biotechnology and Biochemistry*, *69*, 1306–1310.

Lyons, C. L., Finucane, O. F., Murphy, A. M., Cooke, A. A., Viollet, B., Vieira, P. M., et al. (2016). Monounsaturated fatty acids impede inflammation partially through activation of AMPK. *The FASEB Journal*, *30*(Suppl. 1) [296.5].

Maier, T., Fromm, M., Schieber, A., Kammerer, D. R., & Carle, R. (2009). Process and storage stability of anthocyanins and non-anthocyanin phenolics in pectin and gelatin gels enriched with grape pomace extracts. *European Food Research Technology*, *229*, 949–960.

Markov, P. A., Popov, S. V., Nikitina, I. R., Ovodova, R. G., & Ovodov, Y. S. (2011). Anti-inflammatory activity of pectins and their galacturonan backbone. *Russian Journal of Bioorganic Chemistry*, *37*, 817–821.

Marks, D. J., Harbord, M. W., MacAllister, R., Rahman, F. Z., Young, J., Al-Lazikani, B., et al. (2006). Defective acute inflammation in Crohn's disease: A clinical investigation. *Lancet*, *367*, 668–678.

Miró-Casas, E., Covas, M. I., Farre, M., Fito, M., Ortuño, J., Weinbrenner, T., et al. (2003). Hydroxytyrosol disposition in humans. *Clinical Chemistry*, *49*, 945–952.

Muto, E., Dell'Agli, M., Sangiovanni, E., Mitro, N., Fumagalli, M., Crestani, M., et al. (2015). Olive oil phenolic extract regulates interleukin-8 expression by transcriptional and posttranscriptional mechanisms in Cacao-2 cells. *Molecular Nutrition & Food Research*, *59*, 1217–1221.

Nguyen, A. T. B., Winckler, P., Loison, P., & Wache, Y. (2014). Physico-chemical state influences *in vitro* release profile of curcumin from pectin beads. *Colloids and Surfaces B: Biointerfaces*, *121*, 290–298.

Nordkvist, E., Salomonsson, A. C., & Åman, P. (1984). Distribution of insoluble bound phenolic acids in barley grain. *Journal of the Science of Food and Agriculture*, *35*, 657–661.

Pontoniere, P., & Martiradonna, D. (2012). Inflammation and olive polyphenols: a perspective review of supporting literature. *Agro Food Industry Hi Tech*, *23*, 69–71.

Popov, S. V., Markov, P. A., Popova, G. Y., Nikitina, I. R., Efimova, L., & Ovodov, Y. S. (2013). Anti-inflammatory activity of low and high methoxylated citrus pectins. *Biomedicine & Preventive Nutrition*, *3*, 59–63.

Renard, C. M. G. C., Baron, A., Guyot, S., & Drilleau, J. F. (2001). Interactions between apple cell walls and native apple polyphenols: quantification and some consequences. *International Journal of Biological Macromolecules*, *29*, 115–125.

Rodríguez, G., Rodríguez, R., Fernández-Bolaños, J., Guillén, R., & Jiménez, A. (2007). Antioxidant activity of effluents during the purification of hydroxytyrosol and 3,4-dihydroxyphenylglycol from olive oil waste. *European Food Research and Technology*, *224*, 733–741.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., García, A., & Fernández-Bolaños, J. (2015). Novel pectin present in new olive mill wastewater with similar emulsifying and better biological properties than citrus pectin. *Food Hydrocolloids*, *50*, 237–246.

Sánchez-Fidalgo, S., Sánchez de Ibarguen, L., Cardeno, A., & Alarcón de la Lastra, C. (2012). Influence of extra virgin olive oil diet enriched with hydroxytyrosol in a chronic DSS colitis model. *European Journal of Nutrition*, *51*, 497–506.

Sánchez-Fidalgo, S., Cardeno, A., Sánchez-Hidalgo, M., Aparicio-Soto, M., Villegas, I., Rosillo, M. A., et al. (2013). Dietary unsaponifiable fraction from extra virgin

olive oil supplementation attenuates acute ulcerative colitis in mice. *European Journal of Pharmaceutical Sciences*, *48*, 572–581.
Taira, S., Ono, M., & Matsumoto, N. (1997). Reduction of persimmon astrigency by complex formation between pectin and tannins. *Postharvest Biology and Technology*, *12*, 265–271.
Takashima, T., Sakata, Y., Iwakiri, R., Shiraishi, R., Oda, Y., Inoue, N., et al. (2014). Feeding with olive oil attenuates inflammation in dextran sulfate sodium-induced colitis in rat. *The Journal of Nutritional Biochemistry*, *25*, 186–192.
Tsai, P. J., Sun, Y. F., & Hsiao, S. M. (2010). Strengthening the texture of dried guava slice by infiltration of phenolic compounds. *Food Research International*, *43*, 825–830.
Velikov, K. P., (2009). Compositions comprising polyphenol. Patent N° EP2173186A1, Ultrasonics Sonochemistry, 20, 222–231.

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Molecular interactions between 3,4-dihydroxyphenylglycol and pectin and antioxidant capacity of this complex *in vitro*

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ABSTRACT

This study explored the interaction of pectin with 3,4-dihydroxyphenylglycol (DHPG), a potent phenolic antioxidant naturally found in olive fruit, *via* encapsulation into pectinate beads. MALDI TOF-TOF analysis supported the formation of complexes between DHPG and pectin. A combination of covalent bonds (ester bonds) and non-covalent interactions, mostly hydrogen bonding, were suggested as the cause of DHPG-pectin complex formation. Free radical scavenging assays confirmed that DHPG maintained its antioxidant activity after complexation and after a digestion simulated *in vitro* with gastric and intestinal fluids. Therefore, DHPG-pectin beads could reach the large intestine and contribute to a healthy antioxidant environment.

1. Introduction

Virgin olive oil contains numerous antioxidant phenolic compounds that exert potent anti-inflammatory actions (Muto et al., 2015). In the last few years, several studies have demonstrated that diets supplemented with olive oil and/or olive oil phenolics exert a protective effect on experimental colitis in rodents, which may be mediated by their strongly anti-oxidative potential (Sánchez-Fidalgo et al., 2013; Takashima et al., 2014).

3,4-dihydroxyphenylglycol (DHPG) is a simple phenol present in the olive fruit with the same *ortho*-diphenolic structure as hydroxytyrosol (HT), the major phenolic compound in olive fruit, but with an additional hydroxyl group in the β position. DHPG has higher antioxidant, antiradical, and reducing capacity than HT and prevents lipid peroxidation to a degree that is comparable with vitamin E (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007). Furthermore, DHPG is bioavailable, has antioxidant properties in vitamin E-deficient platelet activation and adhesion, and may have anti-inflammatory properties (De Roos et al., 2011).

Pectin is a complex polysaccharide present in the primary cell wall of higher plants. Pectin consists of a homogalacturonan backbone of predominantly α-(1–4)-linked galacturonic acid residues, interrupted by ramified rhamnogalacturonan regions (Schols & Voragen, 1996). The galacturonic acid residues can be methyl-esterified at the carboxyl group, which is essential for the applications of pectins. Pectin, an indigestible soluble fiber, is commonly used in the food and pharmaceutical industries due to its gelling, stabilizer, and thickening properties (Thakur, Singh, & Handa, 1997; Sriamornsak, 2003). Pectin with antimicrobial agent (lysozyme) form composite by electrostatic force and have antibacterial application (Zhang et al., 2015). Pectin also possesses an interesting potential for the delivery of drugs to the colon (Das & Ng, 2010), where it is specifically biodegraded by colonic bacteria and has been found to inhibit both local and systemic inflammation (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Popov et al., 2013).

Our previous study reported the formation of a pectin-HT complex and proposed that HT, DHPG from HT and DHPG-loaded pectinate gel beads, with or without olive oil, reach the colon as agents capable of preventing or improving inflammatory bowel disease (Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017). This study reports the formation of a pectin-DHPG complex and proposes the complex's encapsulation as an efficient system for the delivery of DPHG's antioxidant activity to the colon.

2. Hypotheses

Our previous studies suggested that the formation of a pectin-HT complex promotes a delayed release of HT for a delivery system in the colon region, thus providing a new possibility for treatment of inflammatory disease mediated by oxidative stress.

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The objective of the present investigation is to confirm the presence of a DHPG-pectin complex when the beads based on calcium amidated pectin and calcium pectin-alginate were formed. MALDI TOF-TOF and NMR techniques will study the interactions between pectin and DHPG as well as the efficacy of colon targeting. Also the ability of this phenol-pectin complex to scavenge free radicals, key in the protection against oxidative stress in the colon, will be evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

3. Material and methods

3.1. Materials

DHPG was isolated and purified with a high degree of purity (≥95%) following procedures protected by patent (Fernández-Bolaños Guillén, Jiménez, Rodríguez, Rodríguez-Gutiérrez, & Lama-Muñoz, 2011; Fernández-Bolaños, Rodríguez-Gutiérrez et al., 2014). Highly methylated citrus pectin (degree of methylation 53%), and sodium alginate from brown algae (*Macrocystis pyrifera,* kelp) with 61% mannuronic acid and 39% guluronic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Amidated pectin with a low degree of esterification (30%) and a 21% degree of amidation was a generous gift from Herbstreith & Fox KG (Neuenbürg, Germany). Calcium chloride dihydrate ($CaCl_2$) and a mixture of pectinolytic enzymes from Novozyme Corp. were purchased from Sigma-Aldrich (St Louis, MO, USA).

3.2. Preparation of formulations

Calcium pectin-alginate beads and amidated pectin beads were instantaneously produced by the ionotropic gelation method. 100–300 mg of DHPG (95% of purity) in 30 mL of distilled water with 1.0 g of amidated pectin, or 0.8 g of citrus pectin and 0.2 g of sodium alginate, were homogeneously dispersed using a homogenizer (Ultra-turrax ® T50 Basic, IKA, Germany) at a speed of 24,000 rpm for 5 min in an ice-bath to avoid overheating. Air bubbles were removed from the dispersion by sonication using a bath sonicator. The pectin dispersions containing DHPG, pH 5, were dropped into 75 mL of 10% w/v $CaCl_2$ aqueous solution at room temperature using a nozzle with an inner diameter of 1.2 mm. The $CaCl_2$ solution was gently stirred during hydrogel formation. The beads formed were allowed to stand in the solution for 2 min. DHPG-loaded beads gels were separated from the $CaCl_2$ solution by filtration and washed with distilled water. The filtrated $CaCl_2$ solution and wash liquid were saved to measure the free DHPG that was not entrapped within the beads. The separated gel beads were dried at 37 °C for 48 h in an air-circulated oven until a constant weight was obtained.

3.3. Entrapment efficiency and bioactive compound loading

The entrapment efficiency of the bioactive compound (DHPG) was calculated indirectly by determination of the free DHPG content in the aqueous solution after filtration and washes, according to the equation EE% = [(Qt-Qr)/Qt] x 100, where Qt is the bioactive content initially added during the bead loading, and Qr is the sum of content recovered in the aqueous solution after separating and washing the beads.

The amount of bioactive compound (DHPG) present in the beads (bioactive compound loading, BCL) was also determined by an indirect method according to the equation BCL% = [(Qt-Qr)/Wp] x 100, where Qt is the bioactive content initially added during the bead loading, Qr is the sum of content recovered in the aqueous solution after separating and washing the beads, and Wp is the total weight of dry beads recovered per batch. All experiments were performed in triplicate.

3.4. Extraction and analysis of DHPG from beads

Extraction of DHPG from beads was evaluated in aqueous solutions and various organic solvents. The unbound phenolics were extracted as follows: 0.1 g of dry beads in 100 mL of acidified water for 2 h at 37 °C in a shaking water bath. The quantities of DHPG were determined by HPLC according to a previously published method (Rodríguez et al., 2007). The extraction of strongly bound DHPG was also assayed according to the method of Nordkvist, Salomonsson, and Åman, (1984), using 1 M NaOH containing 0.5% sodium borohydride for 20 min. Also the beads were also subjected to acid hydrolysis according to the method of Graciani & Vázquez (1980). 0.1 g of dry beads/emulsion were treated with 10 mL of 3 N HCl in a homogenizer Ultra-Turrax (24,000 rpm for 5 min) and then heated to 100 °C for 10 min and filtered.

Also 0.1 g of the beads were extracted with 25 mL of organic solvent by stirring (30 min), sonication (15 min), and Ultra-Turrax (24,000 rpm for 5 min), using methanol:water (20, 40, 80%) and dimethyl sulfoxide (DMSO): water (10, 30, 60, 90%). All the mixtures were filtered with filter paper. In no case it was possible to quantify the strongly bound DHPG. Also, due to the difficulty of dissolving the dry beads, the DHPG loaded dried beads were broken down by immersion in sodium phosphate buffer (50 mM, pH 6.8) containing 5 mM of EDTA (Ethylenediaminetetraacetic acid). A preliminary acid treatment was necessary to help the erosion of the matrix (Nguyen, Winckler, Loison, & Wache, 2014): Beads (0.1 g) were dispersed in 100 mL of 0.1 M HCl for 2 h at 37 °C in a shaking water bath. After filtration, the beads were further treated by sodium phosphate buffer with EDTA at 37 °C until dissolution. An alkali extraction ($NaHCO_3$, pH 10) at 100 °C for 30 min was also tested.

3.5. In vitro release study: simulated digestion

The DHPG loaded dried hydrogel beads (0.1 g) were immersed in 100 mL at pH 1.2 with 0.1 M HCl solution (simulated gastric juice) at 37 °C with gentle shaking for the first 2 h, and then in a pH 6.8 sodium phosphate buffer solution (simulated intestinal juice) for the following 2–3 h. The samples were withdrawn and assayed for DHPG content by HPLC.

3.6. Purification of 3,4-dihydroxyphenylglycol-pectin complex

Size exclusion chromatography on two-column Superdex Peptide HR 10/30 (30 × 1 cm) (Pharmacia Biotech, Uppsala, Sweden) connected in line and connected to a Jasco LC-Net II/ADC HPLC (Easton, MD, USA) was used to purify the DHPG-pectin complex. Samples of 1.0 g dried DHPG-loaded amidated pectin beads containing 21.6 mg of DHPG were treated with 100 mL of 0.1 M HCl (pH 1.2) at 37 °C for 2 h to remove all the unbound DHPG and to help break down the beads. Then the beads were dissolved in 100 mL of phosphate buffer (50 mM, pH 6.8) containing 5 mM EDTA and a mixture of pectinolytic enzymes (4 μg/mL) – including *endo*- and *exo*-polygalacturonase and pectinesterase (Novo Nordisk, Bagsvaerd, Denmark). The mixture was incubated at 37 °C for 24 h, then 100 μL was applied ten times onto the column and eluted with distilled water at a flow rate of 0.5 mL/min. The peaks were monitored with a Shodex RI-71 Refraction Index detector. Column calibration was performed with galacturonic acid and tri-galacturonic acid (Fluka) as standards for the pectic oligosaccharides.

3.7. Analysis technique of DHPG-pectin complex

The DHPG-loaded and control beads were analyzed by High Resolution Magic Angle Spinning (HR-MAS). ^1H (500.1 MHz) HR-MAS spectra were recorded on a Bruker Avance-500 spectrometer using $CDCl_3$ as solvent.

MALDI TOF-TOF mass analysis of the isolated fractions of Superdex Peptide HR was performed using an UltrafleXtreme Bruker mass spectrometer Smartbeam-II laser. The MALDI TOF-TOF ms spectra were

acquired in the negative and positive ion mode over a mass-to-charge ratio (*m/z*) range of 150–2000 Da. The instrument was operated at an accelerating voltage of 26.45 kV with an extra voltage of 13.399 kV. Each spectrum was produced by accumulating data from 1000 to 2000 laser shots. The matrix solution of HCCA (alpha-cyano-4-hydroxycinnamic acid) was prepared in 10 mg/mL ACN:H_2O:TFA (50:47.5:2.5) (v/v/v) in the presence of sodium trifluoroacetate. The samples were mixed with the matrix solution in a v/v ratio of 1:100.

3.8. Antiradical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Free radical-scavenging capacity was measured using the DPPH method described in previous work (Rodríguez et al., 2005). The method is based on the measurement of the free radical-scavenging capacity of the DHPG-pectin complex against the stable radical DPPH·. An iMark microplate absorbance reader model 550 (Bio-Rad, Hercules, CA) was used for the absorbance measurements. DPPH· has an absorption band at 515 nm, which disappears upon reduction by antioxidant compound. For each test compound and standard, the decrease in absorbance (expressed as a percentage of the initial absorbance) was plotted against the concentration of the antioxidant in the reaction mixture. The radical-scavenging capacity was tested for different volumes (μL) of each fraction and expressed as percent DPPH·remaining in solution (%DPPH·remaining).

4. Results and discussion

4.1. Preparation of 3,4-dihydroxyphenylglycol (DHPG)-loaded gel beads and in vitro studies of release in gastric and intestinal conditions

Pectin with a low degree of esterification but partially amidated (with amide groups introduced) and citrus pectin with a high degree of esterification combined with sodium alginate in a ratio of 80:20 were used to encapsulate DHPG. The beads were prepared by the emulsion-gelation method using calcium as a counter-ion agent that produces gelation instantaneously by crosslinking the chains.

The phenolic antioxidant 3,4-dihydroxyphenlyglycol (DHPG) used in this study was isolated from olive by-products and purified to 95% purity (Fernández-Bolaños et al., 2011; Fernández-Bolaños, Rodríguez-Gutiérrez et al., 2014). This compound has a high solubility in water although it has also been found in olive oil (Medina, de Castro, Romero, & Brenes, 2006).

The polymeric beads of pectin-alginate and amidated pectin revealed similar entrapment efficacies (EE) of DHPG during the formulation, of 50 and 64%, respectively (Table 1), values relatively high for a highly water soluble compound. The bioactive compound loading (in mg/100 mg of dry beads) increased by more than two-fold when the initial DHPG amount in the pectin-alginate formulation was increased (to 300 mg from 100 mg).

However, an important amount of DHPG was immobilized by interactions with the pectin itself. The binding interaction was strong, resulting in a minimal extraction with acidified methanol (an effective solvent commonly used for the extraction of polyphenols) and in the *in vitro* simulated gastrointestinal tract. The encapsulated DHPG showed a high retention in the *in vitro* dissolution studies from beads in simulated gastric fluid (pH 1.2, 0.1 M HCl solution, 2 h) for the two types of bead preparation used (Table 1). For example, in the case of the pectin-alginate preparation with an initial load of 100 mg DHPG, the retention was 67% retention, which increased to 80% for an initial load of 300 mg. Results from our previous study showed that the course of DHPG release (20–30%) occurred in the first 60 min, when the beads were still apparently intact, although this was slight slower for amidated pectin (Bermúdez-Oria et al., 2017).

A further pH change, simulating intestinal fluid (pH 6.8 phosphate buffer), induced the complete disintegration of the beads (capture of calcium ions by phosphate ion in the medium) but only provided the release of 1–2% of the encapsulated DHPG. Thus the interaction between DHPG and pectin would be strong enough to maintain the complexes even during HPLC determinations. This phenomenon can only be explained by an interaction between DHPG and pectin (amidated and not amidated).

In the case of DPHG-loaded pectin beads, the DHPG retention values (56–80%) were higher than those reported for HT (19–36%) (Bermúdez-Oria et al., 2017), presumably due to the presence of an additional OH group in the DHPG structure, which would allow for a stronger interaction and retention of DHPG, (the pectin-DHPG interactions will be explained further in the next section.) The high retention of DHPG means that a high amount of antioxidant per g of dry beads may be protected from absorption during gastrointestinal transit to reach the colon in significant amounts. For example, from only 100 mg of initial load, it was possible to obtain 12.2 or 23.3 mg of DHPG for amidated and pectin-alginate, respectively, and up to 57.9 mg DHPG/g dry pectin-alginate beads for an initial load of 300 mg (Table 1).

4.2. Investigation of the interaction between DHPG and pectin

In previous work, we suggest the complex formation between HT and pectin by MALDI TOF-TOF analysis (Bermúdez-Oria et al., 2017). In this work, we investigated the interaction between pectin and DHPG, an *ortho*-diphenol similar to HT but with a higher binding percentage. We have demonstrated that this complex was not hydrolysable, not detectable signal by UV absorption and not detectable modification of espectrum by ^{1}H HR-MAS (data not shown). This last result is in agreement with the previous work with HT-pectin complex (Bermúdez-Oria et al., 2017) and the findings by other authors who observed that the complexation of polyphenol with certain classes of polymers, including pectin, was not detectable by ^{1}H NMR (Hayashi, Ujihara, & Kohata, 2005; Velikov, 2009).

Several studies have shown different molecular interactions between specific cell wall polysaccharides and phenolic compounds, proanthocyanidins (Le Bourvellec, Bouchet, & Renard, 2005; Ruiz-Garcia, Smith, & Bindon, 2014), catechin, ferulic acid, and cyanidin-3-glucoside (Phan, Flanagan, D'Arcy, & Gidley, 2017), as well as intermolecular interactions between pectin and proanthocyanidin, a high molecular weight polyphenol (Taira, Ono, & Matsumoto, 1997; Watrelot, Le Bourvellec, Imberty, & Renard, 2013, 2014). Moreover, the persimmon astringency of gallate-type catechin was reduced by complexation with pectin (Hayashi et al., 2005), and pectin was reported to

Table 1
Loading of the bioactive compound (BC) 3,4-dihydroxyphenylglycol (DHPG) in pectin-alginate and amidated pectin beads: entrapment efficiency (EE) and bioactive compounds loading (BCL) in mg/100 mg of dry beads.

Types of beads	Bioactive compound (BC) (mg)		EE% (± SD)	BCL% (± SD)	Retention[a] (%)	mg BC retention/g dry beads
Pectin-Alginate	**DHPG**	100	49.70 ± 0.73	3.45 ± 0.14	67.41 ± 1.92	23.25
		300	60.83 ± 0.94	7.28 ± 0.84	79.50 ± 5.92	57.86
Amidated Pectin	**DHPG**	100	64.16 ± 5.29	2.16 ± 0.34	56.19 ± 5.39	12.21

[a] Retention of BC in simulated gastric fluid (pH 1.2, 0.1 M HCl solution) for 2 h of hydrolytic process and binding capacity (mg BC/g dry beads); SD, experimental n = 2 and analytical n = 3.

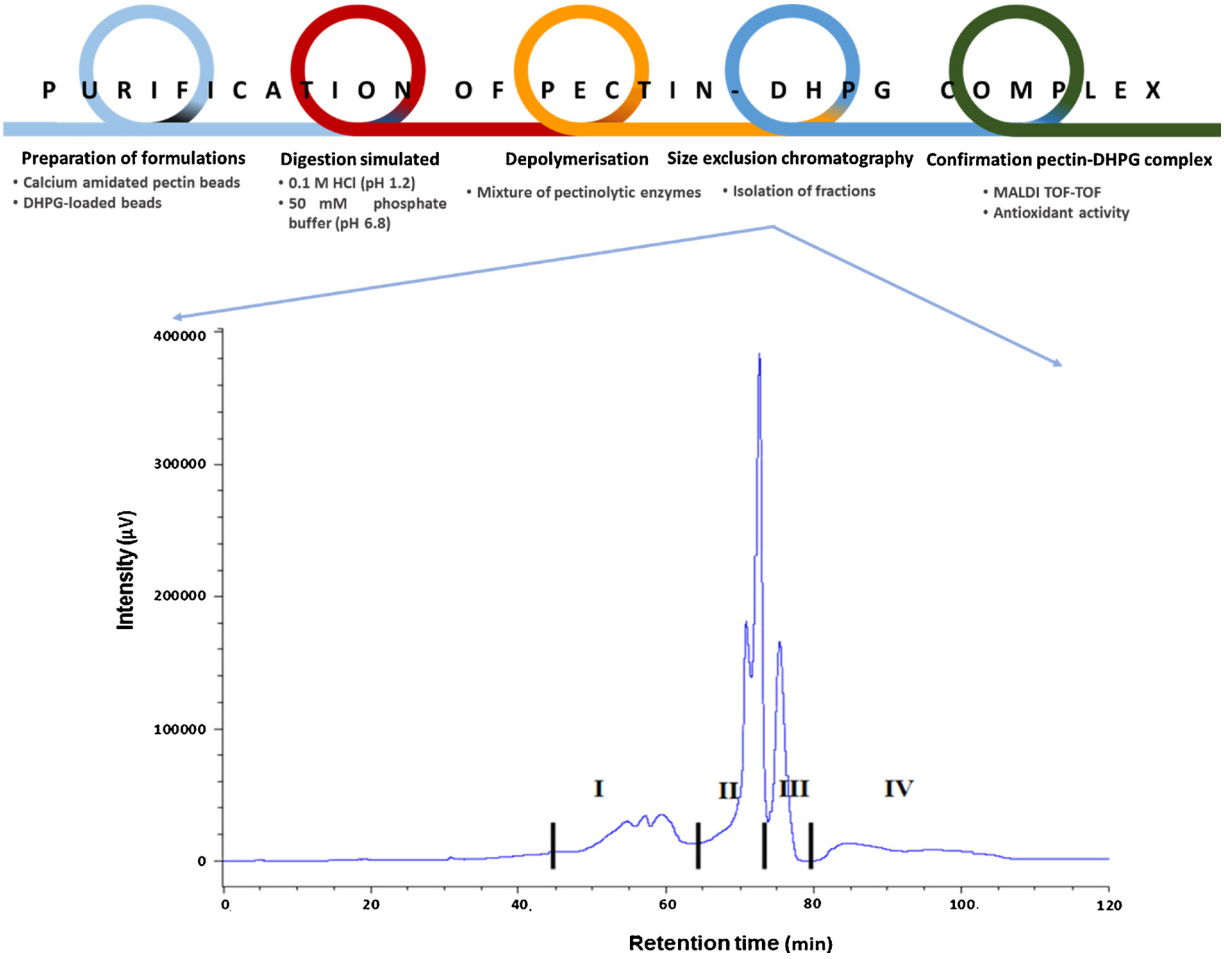

Fig. 1. Scheme of purification procedure for identification of pectin-DHPG complex. Refractive index (RI) elution profiles by size exclusion chromatography using a Superdex Peptide column of the samples of the 3,4-dihydroxyphenylglycol-loaded amidated pectin treated with pectin degrading enzymes. The standard solutions of tri- and monogalacturonic acid had retention times of 52 and 69 min, respectively. Collected fractions were I, II and IV. The peak in fraction III corresponds with the solvent.

stabilize anthocyanins during processing by ionic interactions between the anthocyanin cation and the carboxylic function of the pectin backbone (Maier, Fromm, Schieber, Kammerer, & Carle, 2009). However, the binding mechanism of diverse polyphenols to various cell wall constituents still remains to be addressed.

To provide more information about the DHPG-pectin interaction, an experiment with DHPG encapsulated with calcium amidated pectin beads (21.6 mg DHPG/g dry bead) was carried out. The beads were immersed in simulated gastric fluid at 37 °C for 2 h, filtrated and washed to remove the DHPG unbound, and then in simulated intestinal fluid plus 5 mM EDTA to completely disintegrate the beads. Finally, a mixture of pectinolytic enzymes was added to reduce the degree of polymerization for size exclusion chromatography fractionation in order to provide information about the assumed DHPG-pectin interaction (Fig. 1).

Fractions I, II and IV obtained from amidated pectin and eluted at a retention time of 45–65 min, 65–75 min, and 80–130 min, respectively, were not detectable by UV-absorption, which is indicative on there no free DHPG in these fractions. Therefore, the fractions were analyzed by MALDI TOF-TOF with and without dilution of each fraction (Figs. 2, 3, 5).

After molecular weight distribution followed by refractive index detection, all the possible combinations of different pectin oligosaccharides (Degree Polymerization, DP 1-6) with galacturonic acid units (194 Da, shown in green color) − including amide (193 Da, shown in red color) or methyl-ester (208 Da, shown in blue color) groups and the corresponding possible adducts of sodium − we were unable to compose a structure with almost all the *m/z* values found in the MALDI TOF-TOF spectrum. In many cases, the addition of one or various molecules of DHPG (170 Da, shown in purple color) was necessary to obtain a complex DHPG-pectin oligosaccharide with the corresponding *m/z* values. It is important to remember that sugars (including uronic acid) lose 18 Da (-H_2O) per glycosidic bond.

The MALDI TOF-TOF mass spectra of fractions I and II in positive ion mode are given in Figs. 2 and 3. Both spectra showed a diversity of masses. The ions at *m/z* 1297, 1078, 1057, 1046, 928, 905, 830, and 717 were attributed to oligogalacturonides [M + H^+] composed of tri, tetra, penta or hexamers of galacturonic acid residues, which can be methyl-esterified or amidated at the carboxyl groups and bind with 1, 2 or 3 complete molecules of DHPG (170 Da). Tentative structures of the proposed complexes are summarized in Fig. 4.

Non-covalent interactions (electrostatic forces, hydrogen bonds, and van der Waals forces) formed between DHPG and pectin could explain the strong complex and the high percentage of retention of DHPG (56–80%) of pectinate beads in the simulated gastric and intestinal environment. For example, there are many possible sites of hydrogen bonding between pectin hydroxyl groups and DHPG phenolic OH groups. The presence of the complex between pectin and the *ortho*-diphenol were identified by MALDI TOF-TOF mass spectroscopy and many more pectin fragments associated with DHPG were proposed than in our previous work with HT (Bermúdez-Oria et al., 2017). The additional hydroxyl group that differentiates DHPG from HT could

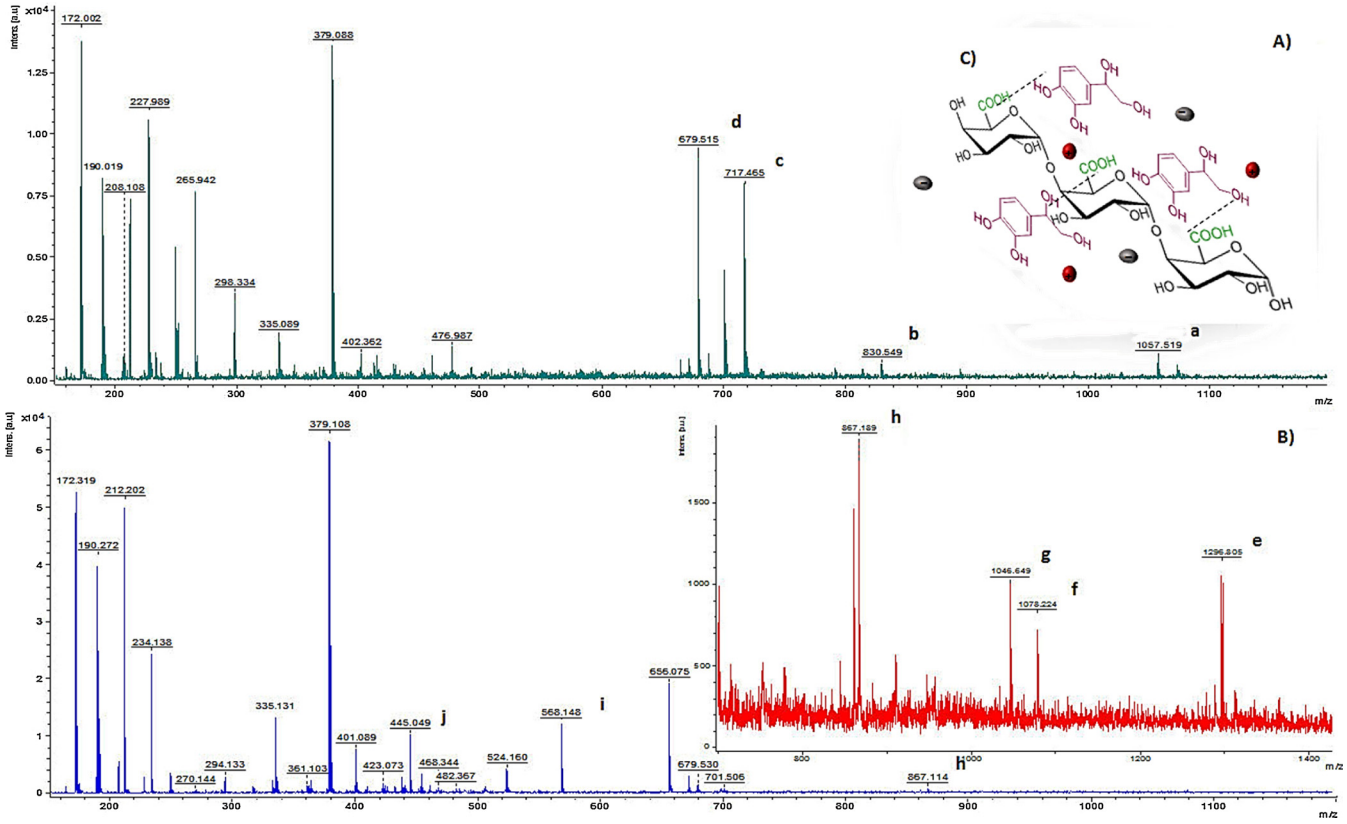

Fig. 2. MALDI-TOF-TOF mass spectra (positive mode) of fraction I **A**) without dilution and **B**) with dilution obtained from amidated pectin treated with pectinolytic enzymes and collected by Superdex Peptide column. In red enlarged spectrum.The signals with *m/z* 190 and 379 correspond to the HCCA test matrix with $[M+H]^+$ + and $[2M+H]^+$, respectively. **C)** Possible binding interactions between DHPG (in purple) and pectin, corresponding to tentative structure of signal *m/z* at 1057. The lower case letter a, b, c, d, e, f, g, h, I and j are explained en Fig. 4, fraction I, 2A(a), 2A(b), 2A(c), 2A(d) and 2B(e), 2B(f), 2B(g), 2B(h), 2B(i), 2B(j). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contribute to more H-bond formation, and thus explain the increased bead retention and higher abundance of the complexes in MALDI TOF-TOF analysis.

Phenolic compounds have both a hydrophobic aromatic ring and hydrophilic hydroxyl groups with the ability to bind to specific polysaccharides of the cell wall. Non-covalent interactions, such as electrostatic and dipolar forces, hydrogen bonds, and/or van der Waals attractions, were also suggested for the association between polyphenols and dietary fiber (Nantitanon, Yotsawimonwat, & Okonogi, 2010; Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011). The precise type of dietary fiber-polyphenol association (covalent or non-covalent bond) remains to be elucidated. In the reported case of procyanidin-pectin binding, the linkage was strong due to charge interactions and/or the presence of hydrophobic cavities in the pectin gel (Le Bourvellec & Renard, 2012). Also the hydrogen bond between the hydroxyl group of phenolic compounds and the oxygen atom of the polysaccharide's glycosidic linkage may contribute to binding interactions (Quirós-Sauceda et al., 2014). The possible interactions between DHPG-pectin oligosaccharides are illustrated in Figs. 2 and 3.

In fractions I and II, common ions also appeared at *m/z* 701, 679, 568 (previously described by Van Alebeek. Schols, & Voragen, 2000), and 445, which correspond with oligogalacturonides resulting from the enzymatic digestion of pectin, partially methyl-esterified or amidated with a degree of polymerization of 2–3, and/or a fragment described by Domon and Costello (1988) that corresponds to a cross-ring of–60 Da of the non-reducing end or 133 Da if it contains the reducing end of an amidated galacturonic acid-type. All of these signals correspond to fragments of pectin without the presence of DHPG.

Curiously, for fraction IV, which eluted after the solvent due to certain interactions with the Superdex Peptide HR, signals common to fractions I and II appeared, such as *m/z* 568 and 445, identified as oligogalacturonides, and other peaks that may be fragments of unidentified pectin (Fig. 5). However, four signals were exclusive to fraction IV, at *m/z* 877, 522, 482, and 468 (marked with circles, Fig. 5). Two of these ions, at *m/z* 877 and 522, were proposed to be esters of DHPG. In the sample acquired with dilution, the signal at *m/z* 877 would be composed of a dimer methyl esterified galacturonic acid-type and a galacturonic acid-build unit with a fragment ion $[M+H]^+$ at *m/z* 385, covalently linked with DHPG by an ester bond. The acid group would form an ester group with a DHPG molecule (170 Da) for the given mass *m/z* 537, and loss of a molecule of H_2O (−18 Da), and two additional complete molecules of DHPG (170 Da) would make a possible complex of esterified-pectin-dimer-DHPG with *m/z* 877. Signal c) at *m/z* 522, may be attributed to a dimer composed of amidated galacturonic acid-type build units with a fragment ion $[M+H]^+$ at *m/z* 369, covalently linked with DHPG (170 Da) by ester bond to give *m/z* 522, with a difference of 17 *m/z* units attributable to the loss of an $-NH_3$ group. The formation of this covalent bonds through NH_2 of amide group is similar to covalent interaction described between primary amine group of proteins and aminoacids and phenolic compounds in the form either as phenolic radical or quinones (Iacomino et al., 2017; Prigent, Visser, van Koningsveld, & Gruppen, 2007).

The presence of dimers of pectin covalently linked to DHPG as an ester, and one with DHPG in a non-covalently linked complex is in agreement with the results of Fernández-Bolaños, Rubio-Senent, Lama-Muñoz, García, and Rodríguez-Gutiérrez (2014), who observed glucosides attached to hydroxytyrosol and tyrosol that interacted with the Superdex-Peptine HR and eluted after the solvent. Similar results with feruloyl oligosaccharides were described in plant cell wall of bamboo (Ishii, 1991) and sugarbeet (Micard, Renard, Colquhoun, & Thibault, 1997).

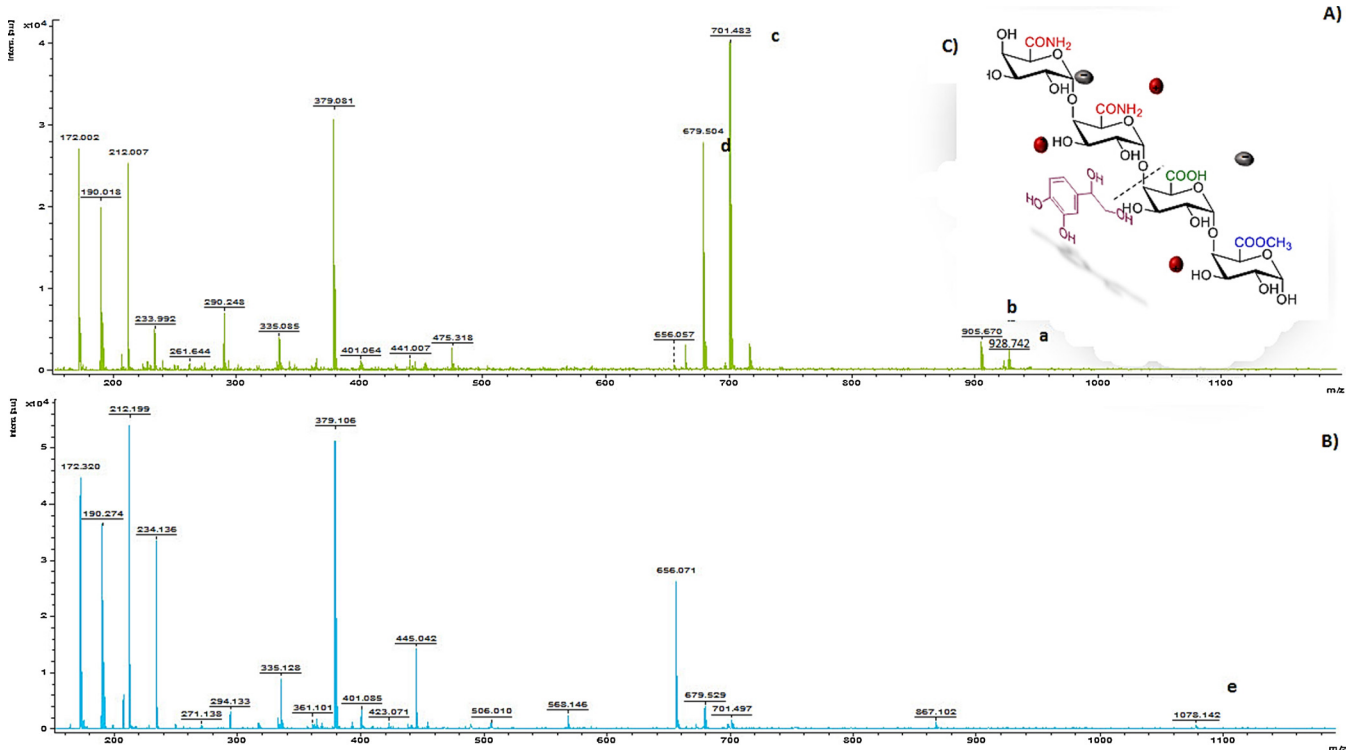

Fig. 3. MALDI-TOF-TOF mass spectra (positive mode) of fraction II **A**) without dilution and **B**) with dilution obtained from amidated pectin treated with pectinolytic enzymes and collected by Superdex Peptide column. The signals with *m/z* 190 and 379 correspond to the HCCA test matrix with $[M + H]^+$ + and $[2M + H]^+$, respectively. **C)** Possible binding interactions between DHPG (in purple) and pectin, corresponding to tentative structure of signal *m/z* at 905. The lower case letter a, b, c, d and e are explained en Fig. 4, fraction II, 3A(a), 3A(b), 3A(c), 3A(d) and 3B(e).

In this context, recent studies have shown that phenolic compounds are able to interact and bind with cellulose, pectin, and other indigestible components of plant cell walls. Different molecular interactions between the dietary fiber of fruits and vegetables and polyphenols occurred during consumption or processing (Padayachee, Day, Howell, & Gidley, 2017). These interactions may have an impact on the delivery and absorption *in vivo* of the polyphenols, where most ingested polyphenols are not bioaccessible due to gastric and small intestinal digestion (Correa-Betanzo, Padmanabhan, Corredig, Subramanian, & Paliyath, 2015). This means that if the bounds are enough strong polyphenols could reach the colon where they would be released and metabolized by the action of microbiota and, in part, reabsorbed.

It is essential to understand the influence of the food matrix on the release of functional components, and the role of dietary fiber as a delivery system of bioactive compounds must be studied in more depth (Quirós-Sauceda et al., 2014). However, the results obtained in this work suggest that the linkage formed between the available hydroxyl groups of DHPG and the pectin of calcium pectinate beads plays an important role in transporting this antioxidant through the upper gastrointestinal tract to the colon.

4.3. Antioxidant activity in DHPG-pectin complex

The ability to scavenge DPPH radicals was assessed for fractions I, II, III (solvent), and IV obtained from DHPG-amidated pectin beads after in vitro simulated conditions of the gastrointestinal tract, following an enzymatic treatment and separation by size exclusion chromatography. Fractions I and II, with several DHPG-pectin (hexamer, pentamer, tetramer, and trimer) complexes, showed a clear increase in DPPH scavenging ability with respect to fraction III, which corresponds to the solvent peak without antioxidant activity (Fig. 6). The DHPG-pectin complex still retains some antioxidant activity because the whole phenolic compound is not compromised, as occurred in the case of the HT-pectin complex, for which no antioxidant activity was detected (Bermúdez-Oria et al., 2017). DHPG has an additional −OH group with respect to HT, and this greater availability of free hydroxyl groups on the aromatic ring is responsible for the complex's antioxidant activity. Possibly, this is also why fraction IV presents higher antiradical activity, due to the formation of an ester between the polygalacturonic acid moieties of pectin and the hydroxyl group of the lineal chain of DHPH, leaving the key hydroxyl groups of DHPG free.

These interactions may have an impact on the delivery and absorption of phenol *in vivo*, but they may be protected during gastrointestinal transit to the colon. The presence of significant amounts of antioxidant in the colon in free form after fermentation by the microflora, or even in complexed form with pectin, could be of importance considering it would reduce the oxidative stress *in situ* and thus decrease the risk of inflammatory diseases of the colon (Quirós-Sauceda et al., 2014).

5. Conclusions

This work suggests that DHPG, a natural phenol present in olive fruit, interacts with pectin to form complexes. The antioxidant activity of these complexes was demonstrated *in vitro* by the DPPH assay. These interactions could be due to a combination of covalent bonds (ester bonds) and non-covalent bonds (hydrogen bonding and electrostatic interactions) as suggested by MALDI TOF-TOF analysis. The presence of dimers of pectin covalently linked to DHPG as an ester *via* group acid or amide of pectin could explain in part the strong binding. However, this would not explain the increased beads retention of DHPG respect to HT or the higher retention of pectin-alginate respect to pectin amidated. A complete understanding on the formation of phenolic-pectin covalent or non-covalent adduct will be addressed in future work.

Complex formation between the natural antioxidant DHPG and pectin *via* encapsulation protects the DPHG from degradation during

Fig. 4. Tentative structures of the proposed DHPG-pectin complex.

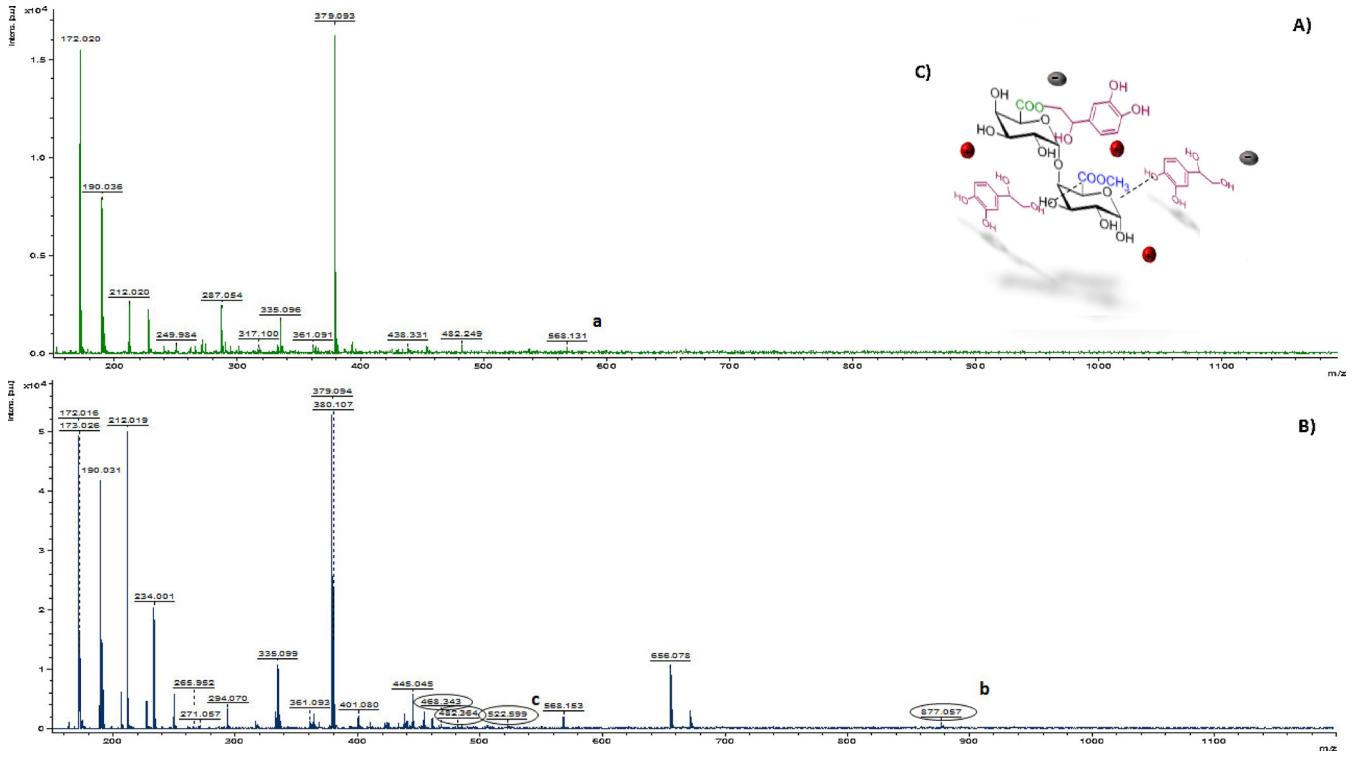

Fig. 5. MALDI-TOF-TOF mass spectra (positive mode) of fraction IV **A)** without dilution and **B)** with dilution obtained from amidated pectin treated with pectinolytic enzymes and collected by Superdex Peptide column. The signals with *m/z* 190 and 379 correspond to the HCCA test matrix with $[M + H]^+$ + and $[2M + H]^+$, respectively. **C)** Possible binding interactions between DHPG (in purple) and pectin, corresponding to tentative structure of signal *m/z* at 877. The lower case letter a, b and c are explained en Fig. 4, fraction IV, 5A(a), 5B(b), 5B(c).

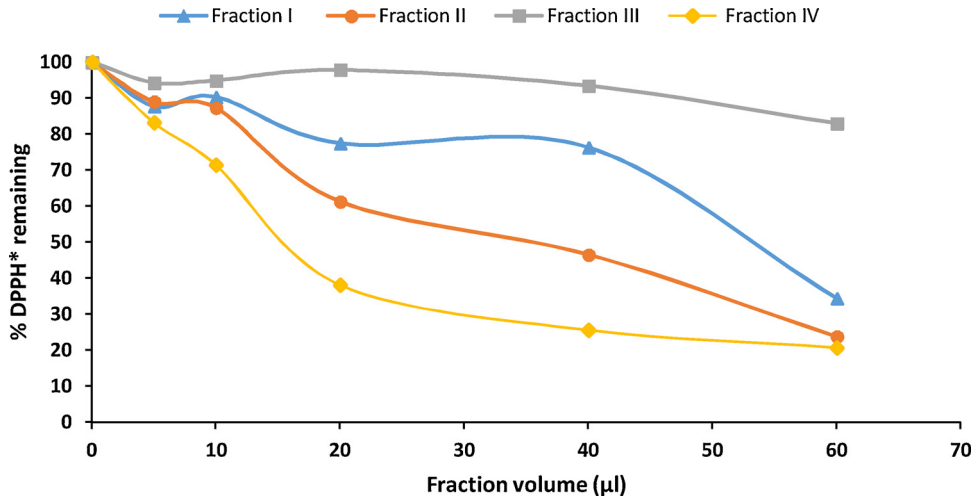

Fig. 6. DPPH assay to measure the antiradical capacity of different fractions of 3,4-dihydroxyphenylglycol-loaded amidated pectin treated with pectinolytic enzymes and obtained by size exclusion chromatography.

gastrointestinal transit to the colon. The subsequent release of bioactive DPHG molecules in situ can have significant beneficial effects on intestinal health to prevent or improve IBD.

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References

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Lama-Muñoz, A., & Fernández-Bolaños, J. (2017). Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting. *Carbohydrate Polymers, 163*, 292–300.

Correa-Betanzo, J., Padmanabhan, P., Corredig, M., Subramanian, J., & Paliyath, G. (2015). Complex formation of blueberry (Vaccinium angustifolium) anthocyanins during freeze-drying and its influence on their biological activity. *Journal of Agricultural and Food Chemistry, 63*, 2935–2946.

Das, S., & Ng, K. Y. (2010). Colon-specific delivery of resveratrol: Optimization of multi-particulate calcium-pectinate carrier. *International Journal of Pharmaceutics, 385*, 20–28.

De Roos, B., Zhang, X., Rodríguez Gutiérrez, G., Wood, S., Rucklidge, G. J., Reid, M. D., et al. (2011). Anti-platelet effects of olive oil extract: In vitro functional and

proteomic studies. *European Journal of Nutrition, 50*, 553–562.
Domon, B., & Costello, C. E. (1988). Structure elucidation of glycosphingolipids and gangliosides using high-performance tandem mass-spectrometry. *Biochemistry, 27*, 1534–1543.
Fernández-Bolaños, J., Guillén, R., Jiménez, A., Rodríguez, R., Rodríguez-Gutiérrez, G., & Lama-Muñoz, A. (2011). *Method for purifying 3,4-dihydroxyphenylglycol (DHPG) from plant products. International publication number WO 2010/070168*.
Fernández-Bolaños Guzmán, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Rubio-Senent, F., Fernández-Bolaños Guzmán, J. M., Maya, I., et al. (2014). *Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroyphenylglycol extract, and hydroxytyrosol acetate extract, from by-products of the olive tree, and the purification thereof. International publication number WO 2013/007850*.
Fernández-Bolaños, J., Rubio-Senent, F., Lama-Muñoz, A., García, A., & Rodríguez-Gutiérrez, G. (2014). Production of oligosaccharides with low molecular weights secoiridoids and phenolic glycosides from thermally treated olive by-products. In L. S. Schqeizer, & S. J. Krebs (Eds.). *Oligosaccharides: Food sources, biological roles and health implications* (pp. 173–208). New York: Nova Science Publishers.
Graciani, E., & Vázquez, A. (1980). Estudio de los componentes del aceite de oliva por cromatografía líquida de alta eficacia (HPLC). II Cromatografía en fase inversa. *Grasas Y Aceites, 31*, 237–243.
Hayashi, N., Ujihara, T., & Kohata, K. (2005). Reduction of catechin astrigency by the complexation of gallate-type catechins with pectin. *Bioscience Biotechnology and Biochemistry, 69*, 1306–1310.
Iacomino, M., Weber, F., Gleichenhagen, M., Pistorio, V., Panzella, L., Pizzo, E., et al. (2017). Stable benzacridine pigments by oxidative coupling of chlorogenic acid with amino acids and proteins: Toward natural product-Based green food coloring. *Journal of Agricultural and Food Chemistry, 65*, 6519–6528.
Ishii, T. (1991). Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-walls. *Carbohydrate Research, 219*, 15–22.
Le Bourvellec, C., & Renard, C. M. (2012). Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. *Critical Reviews in Food Science and Nutrition, 52*, 213–248.
Le Bourvellec, C., Bouchet, B., & Renard, C. M. (2005). Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochimica et Biophysica Acta-General Subjects, 1725*, 10–18.
Maier, T., Fromm, M., Schieber, A., Kammerer, D. R., & Carle, R. (2009). Process and storage stability of anthocyanins and non-anthocyanin phenolics in pectin and gelatin gels enriched with grape pomace extracts. *European Food Research Technology, 229*, 949–960.
Markov, P. A., Popov, S. V., Nikitina, I. R., Ovodova, R. G., & Ovodov, Y. S. (2011). Anti-inflammatory activity of pectins and their galacturonan backbone. *Russian Journal of Bioorganic Chemistry, 37*, 817–821.
Medina, E., de Castro, A., Romero, C., & Brenes, M. (2006). Comparison of the concentrations of phenolics compounds in olive oil and other plant oils: Correlation with antimicrobial activity. *Journal of Agricultural and Food Chemistry, 54*, 4954–4961.
Micard, V., Renard, C. M. G. C., Colquhoun, I. J., & Thibault, J. F. (1997). End-products of enzymic saccharification of beet pulp, with a special attention to feruloylated oligosaccharides. *Carbohydrate Polymers, 32*, 283–292.
Muto, E., Dell'Agli, M., Sangiovanni, E., Mitro, N., Fumagalli, M., Crestani, M., et al. (2015). Olive oil phenolic extract regulates interleukin-8 expression by transcriptional and posttranscriptional mechanisms in Cacao-2 cells. *Molecular Nutrition & Food Research, 59*, 1217–1221.
Nantitanon, W., Yotsawimonwat, S., & Okonogi, S. (2010). Factors influencing antioxidant activities and total phenolic content of guava leaf extract. *LWT – Food Science and Technology, 43*, 1095–1103.
Nguyen, A. T. B., Winckler, P., Loison, P., & Wache, Y. (2014). Physico-chemical state influences in vitro release profile of curcumin from pectin beads. *Colloids and Surfaces B: Biointerfaces, 121*, 290–298.
Nordkvist, E., Salomonsson, A. C., & Åman, P. (1984). Distribution of insoluble bound phenolic acids in barley grain. *Journal of the Science of Food and Agriculture, 35*, 657–661.
Padayachee, A., Day, L., Howell, K., & Gidley, M. J. (2017). Complexity and health functionality of plant cell wall fibers from fruits and vegetables. *Critical Reviews in Food Science and Nutrition, 57*, 59–81.
Palafox-Carlos, H., Ayala-Zavala, J. F., & González-Aguilar, G. A. (2011). The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *Journal of Food Science, 76*, R6–R15.
Phan, A. D. T., Flanagan, B. M., D'Arcy, B. R., & Gidley, M. J. (2017). Binding selectivity of dietary polyphenols to different plant cell wall components: Quantification and mechanism. *Food Chemistry, 233*, 216–227.
Popov, S. V., Markov, P. A., Popova, G. Y., Nikitina, I. R., Efimova, L., & Ovodov, Y. S. (2013). Anti-inflammatory activity of low and high methoxylated citrus pectins. *Biomedicine & Preventive Nutrition, 3*, 59–63.
Prigent, S. V. E., Voragen, A. G. J., Visser, A. J. W. G., Koningsveld, G. A., & Gruppen, H. (2007). Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *Journal of the Science of Food and Agriculture, 87*, 2502–2510.
Quirós-Sauceda, A. E., Palafox-Carlos, H., Sayago-Ayerdi, S. G., Ayala-Zavala, J. F., Bello-Perez, L. A., Alvarez-Parrilla, E., et al. (2014). Dietary fiber and phenolic compounds as functional ingredients: Interaction and possible effect after ingestion. *Food & Function, 5*, 1063–1072.
Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández- Bolaños, J., et al. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. *Journal of Agricultural and Food Chemistry, 53*, 5212–5217.
Rodríguez, G., Rodríguez, R., Fernández-Bolaños, J., Guillén, R., & Jiménez, A. (2007). Antioxidant activity of effluents during the purification of hydroxytyrosol and 3,4-dihydroxyphenylglycol from olive oil waste. *European Food Research and Technology, 224*, 733–741.
Ruiz-Garcia, Y., Smith, P. A., & Bindon, K. A. (2014). Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls. *Carbohydrate Polymers, 114*, 102–114.
Sánchez-Fidalgo, S., Cardeno, A., Sánchez-Hidalgo, M., Aparicio-Soto, M., Villegas, I., Rosillo, M. A., et al. (2013). Dietary unsaponifiable fraction from extra virgin olive oil supplementation attenuates acute ulcerative colitis in mice. *European Journal of Pharmaceutical Sciences, 48*, 572–581.
Schols, H. A., & Voragen, A. G. J. (1996). Complex pectins: Structure elucidation using enzymes. *Pectins and Pectinases, 14*, 3–19.
Sriamornsak, P. (2003). Chemistry of pectin and its pharmaceutical uses: A review. *Silpakorn University International Journal, 3*, 206–228.
Taira, S., Ono, M., & Matsumoto, N. (1997). Reduction of persimmon astringency by complex formation between pectin and tannins. *Postharvest Biology and Technology, 12*, 265–271.
Takashima, T., Sakata, Y., Iwakiri, R., Shiraishi, R., Oda, Y., Inoue, N., et al. (2014). Feeding with olive oil attenuates inflammation in dextran sulfate sodium-induced colitis in rat. *The Journal of Nutritional Biochemistry, 25*, 186–192.
Thakur, B. R., Singh, R. K., & Handa, A. K. (1997). Chemistry and uses of pectin–a review. *Critical Reviews in Food Science and Nutrition, 37*, 47–73.
Van Alebeek, G.-J. W. M., Schols, H. A., & Voragen, A. G. J. (2000). Amidation of methylesterified oligogalacturonides: Examination of the reaction products using MALDI-TOF MS. *Carbohydrate Polymers, 46*, 311–321.
Velikov, K. P. (2009). Compositions comprising polyphenol. Patent No. EP2173186A1.
Watrelot, A. A., Le Bourvellec, C., Imberty, A., & Renard, C. M. G. C. (2013). Interactions between pectic compounds and procyanidins are influenced by methylation degree and chain length. *Biomacromolecules, 14*, 709–718.
Watrelot, A. A., Le Bourvellec, C., Imberty, A., & Renard, C. M. G. C. (2014). Neutral sugar side chains of pectins limit interactions with procyanidins. *Carbohydrate Polymers, 99*, 527–536.
Zhang, T., Zhou, P., Zhan, Y., Shi, X., Lin, J., Du, Y., et al. (2015). Pectin/lysozyme bilayers layer-by-layer deposited cellulose nanofibrous mats for antibacterial application. *Carbohydrate Polymers, 117*, 687–693.

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ORIGINAL RESEARCH

Anti-Inflammatory Local Effect of Hydroxytyrosol Combined with Pectin-Alginate and Olive Oil on Trinitrobenzene Sulfonic Acid-Induced Colitis in Wistar Rats

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ABSTRACT

Purpose: Evaluate the efficacy of hydroxytyrosol in the local treatment of inflammatory colitis. Currently, the existing treatments for inflammatory bowel diseases does not cure the disease and it is associated with high rates of side effects and complications. Hydroxytyrosol is a phenyl-ethyl-alcohol derived from the hydrolysis of oleuropein and present in olive oil, previous studies have demonstrated the anti-inflammatory effect of dietary hydroxytyrosol supplement, with no toxicity. *Materials & Methods:* Colitis has been induced by using Trinitrobenzene Sulfonic Acid at 40 rats. They were divided into four groups randomly: 10 rats without treatment; 10 rats with pectin/alginate mixture; 10 rats treated with pectin/alginate + olive oil; 10 rats treated with pectin/alginate + olive oil + hydroxytyrosol. Animals were sacrificed 10 days after induction of trinitrobenzene sulfonic acid, receiving 5 days of continuous treatment. Samples of the rectal area were studied and observed under a microscope to determine the damage by Hunter scoring modified, assessing inflammatory infiltration, number of intestinal walls involved, damage to the mucosal architecture, and edema. *Results*: When the rectum was analyzed in a global way, nonsignificant differences were observed; however, when performing an individualized analysis, statistically significant differences in the inflammatory infiltrate are present in the samples, which were evaluated using the ANOVA and Student-T statistics. *Conclusions*: Local treatment with the natural antioxidant hydroxytyrosol combined with pectin/alginate and olive oil of inflammatory bowel disease has been shown to be effective against inflammatory infiltration of TNBS-induced colitis.

Keywords: colitis; hydroxytyrosol; inflammatory bowel disease; olive oil; pectin/alginate

1. INTRODUCTION

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases characterized by a diffuse inflammation of the colon with a relapse and remitting course, and particularly common in developed countries.[1,2] Although the specific causes are not completely understood, it involves a combination of genetic, environmental, and immunologic factors [3] which regulates the synthesis and release of different proinflammatory mediators, including active oxygen and nitrogen metabolites, eicosanoids, platelet-activating factor, and cytokines. The pathogenic

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cascade is initiated which, in turn, perpetuates the inflammatory response in the bowel due to these mediators.[4]

Treatment of IBD remains a challenge, it is directed to intestinal inflammation with the intention of altering the natural history of the disease, and current therapies include 5-aminosalicylates, sulfasalazine, antibiotics, glucocorticoids, and immunomodulators. Unfortunately, the long-term uses of these agents with high dosage are associated with serious adverse effect and compromise the therapeutic advantage.[5] Besides the medical treatment, a high percentage close to 70% require surgery, with the morbimortality that this entails.[6] Therefore, there is growing interest for alternative remedies for treating the IBD.

In the last few years, several studies demonstrated that diets supplemented with olive oil and/or olive oil phenolics compounds exert a protective effect in experimental colitis in rodents, which may be mediated by their strongly antioxidative potential.[7,8]

Evidence shows that virgin olive oil might exert beneficial effects on markers of inflammation.[9] The oil's high monounsaturated fatty acid (MUFA) content and the presence of minor components such as erythrodiol, β-sitosterol, squalene, tocopherols, carotenoids, and phenolic compounds, exert the anti-inflammatory effect.[10] Considering the involvement of oxidative stress in inflammation, antioxidants might bring benefits in inflammatory diseases. In fact, virgin olive oil contains numerous antioxidant phenolic compounds that exert potent anti-inflammatory actions.[11]

Hydroxytyrosol (HT) is a simple phenol and considered to be the main olive oil antioxidant with an exceptional free radical scavenger capacity, and also possesses a potent anti-inflammatory activity.[12,13] The European Food Safety Authority (EFSA) allows the health claim that ≥ 5 mg HT/day prevents low-density lipoprotein (LDL) oxidation and, therefore, reduces the risk of atherosclerosis.[14] Thus, it is an indirect recognition of the anti-inflammatory effect of HT.[15]

Pectin is naturally present in plant cell walls, it is specifically biodegraded by colonic bacteria, and has been found to inhibit both local and systemic inflammation and prevent intestinal inflammation.[16,17]

The present study was designed to evaluate the effectiveness of local treatment with hydroxytyrosol in combination with pectin/alginate and olive oil on colon inflammation during the colitis induced in rats. Secondary objectives are to assess the feasibility of the procedure; check the performance of different treatments; observe whether the pectin/alginate-olive oil mixture may exert any protective effect against acute or chronic intestinal disorders; to evaluate the histological changes that have occurred after the application by enema of the drug, comparing it between the different groups, and to evaluate the safety of the treatment in the short term.

2. MATERIAL AND METHODS

2.1. Materials

Hydroxytyrosol was extracted and purified from olive byproducts using a chromatographic system. Citrus pectin with a high degree of esterification (53%), and sodium alginate were purchased from Sigma-Aldrich (St Louis, MO, USA). Virgin olive oil was purchased from a local supermarket.2,4,6-trinitrobenzenesulfonic acid (TNBS) was also purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Preparation of Formulations

Citrus pectin (0.8 g) and sodium alginate (0.2 g), were homogeneously dispersed in 30 mL of water. 7.5 g of virgin olive oil was added to the polymers, and the mixture was emulsified using a homogenizer (Ultra-Turrax ® T50 Basic, IKA, Germany) at a speed of 24,000 rpm for 5 min. In the case of the incorporation of hydroxytyrosol (90% of purity), an aqueous solution containing this antioxidant was added to pectin-alginate and olive oil and the mixture was emulsified using the same homogenization conditions. The proportion was 40 mg of HT in every 2 ml of final mixture.

The mixture should be preserved at −20°C until its first use. Between short periods of use, the emulsion will remain at 4 ºC.

2.3. Animals and Study Design

A total of 40 female Wistar rats, whose mean weight was of 248.5 g (the weights oscillated between 205 and 294 g), were used in this study. Rats were randomized into four treatment groups after performing a simple randomization using the program "Epidad v.4": the first group (10 animals) was "Control group" with no administration of any treatment. The second group or "Group A" (10 animals) received a treatment of pectin/alginate. The third group or "Group B" (10 animals) received a treatment of a mixture of pectin/alginate and virgin olive oil. The fourth group or "Group C" (10 animals) received a treatment composed of pectin/alginate plus virgin olive oil and hydroxytyrosol (HT). Previously, the animals were infected by intrarectal induction with TNBS. In the groups of animals A, B, and C, the formulations were administrated daily for 5 days after the TNBS colitis was induced (3 days after administration of TNBS), and the animals were sacrificed 3 days after finishing the treatment. To allow acclimatization to the environment, the animals were housed a week before the experiment with unrestricted access to diet and water. The feed consisted of "standard" feed for laboratory

TABLE 1 Hunter's score.

Hunter's score	Rank
Inflammatory infiltration	(0–3)
Number of involved bowel walls	(0–3)
Damage to the mucosal architecture	(0–1)
Edema	(0–1)

The value 0 symbolizes the nonexistence of damage. On the scale from 0 to 3, there are different degrees of damage. In the scale that goes from 0 to 1 means that there is no damage if the value is 0 or there is damage if the value is 1.

animals with energy value of 2900 calories/kg. The 12 hours before starting the procedure, the rats must remain fasting.

2.4. Induction of Colitis by TNBS and Treatment Protocol

Animals were anesthetized (ketamine:xylazine 80:10 mg/kg) by intraperitoneal injection, and colitis induced by intrarectal instillation of a solution 1 mL of diluted TNBS (0.5 mL TNBS + 0.5 mL of physiological serum), using a flexible catheter via anus. Animals were held in Tredelenburg´s position for 15 min after the installation to ensure the permanence of the TNBS solution into the colon. During the next 3 days, the animals stayed with free water and feed "ad libum" again. After this period, once induced the colitis, every group of animals previously sedated received a dose of 2 ml of each different formulation during 5 days. During this same period, the animal received a 1 mg/kg dose of the analgesic Meloxican (AINE) by subcutaneous injection every 24 h.

Three days after the end of the treatment, or 10 days after the induction of TNBS, that it is the same, the animals were sacrificed. Diluted sodium pentobarbital (10 mg/kg) administered by intraperitoneal injection was used for the slaughter of the animal. Rectal samples were taken. Samples were cut using a microtome at a thickness of 4 micrometers and stained using Hematoxylin/Eosin. After all this process, the samples were evaluated based on the modified Hunter score as explained in Table 1.[18]

2.5. Assessment of Colitis

Rats were examined daily for signs of disease and body weight change.

The animals were sacrificed and the rectum was removed for assessment, and dyed and stained with hematoxylin and eosin. Histopathology was assessed in a blinder fashion using an 8-point scale according to a modified Hunter´s score (Table 1).[18]

TABLE 2 Homogeneity between weights of rats.

Groups	Levene	Significance (p)
Control – C	Induction day weight	0.622
	Post-treatment weight	0.524
	Weight difference	0.410
A – B	Induction day weight	0.538
	Post-treatment weight	0.931
	Weight difference	0.091
B – C	Induction day weight	0.415
	Post-treatment weight	0.910
	Weight difference	0.949
Control – A	Induction day weight	0.878
	Post-treatment weight	0.641
	Weight difference	0.461

This table shows the values of significance between groups taking into account the values of weights of the rats on the day of induction, after the end of the treatment and with the difference between the weights of the rats between the groups.

2.6. Ethical Statement

The animals have been treated throughout the experimental process according to the Council of Europe agreements on the protection of animals used in animal experiments.

The protocol adopted for this study was approved by the Ethic Committee of Virgen del Rocío Hospital and by the Animal Experimental Committee of Consejería de Agricultura y Pesca de la Junta de Andalucía.

As end-point criteria, the typical signs of pain and distress were taken into account. With the presence of two of them, it would advise the re-evaluation of the analgesia that is being performed in the post-operative period, and the presence of five of them would force the euthanasia of the rat through the injection of a Ketamine-Diazepam IP overdose sedation with Halothane. Also, as the procedure was abdominal, we took special interest in the monitoring of anorexia, vomiting, and abnormal or protective postures.

2.7. Statistical Analysis

Statistical studies were performed to verify homogeneity between groups using the Levene test, using the collected data of the weights before and after the process that the animals have passed through (Table 2). Results were expressed as mean values ± standard deviations. STATGRAPHICS® plus software and IBM® SPSS® Statistics 19 were used for statistical analysis. Comparisons among groups were made using one-way analysis of variance (ANOVA) and Student's t-test methods. The comparisons between groups were made by pair. A p-value less than 0.05 was considered significant.

TABLE 3 Hunter score of rectum of each group.

	Group Control	Group A	Group B	Group C
Inflammatory infiltration (0–3)	2.0	1.4	1.2	0.2
Number of involved bowel walls(0–3)	1.5	1.3	1.0	1.1
Damage to the mucosal architecture (0–1)	0.7	0.5	0.4	0.6
Edema (0–1)	0.0	0.2	0.0	0.0
TOTAL SCORE ((0–8) ± standard deviation)	4.2 ± 3.406	3.4 ± 2.633	2.6 ± 1.364	1.9 ± 2.394

There are significant differences in the inflammatory infiltration parameter between group C and the rest of the groups. The significance value when group C was compared with the other groups was less than $p < 0.05$. For Control group the inflammatory infiltration observed was moderate (2.0), for groups A (1.4) and B (1.2) the inflammatory infiltration was mild, and no histopathological alterations were observed in group C (0.2).

3. RESULTS

The complete procedure could be carried out successfully in all the rats included in the study, so, we conclude that the feasibility of the experiment was 100%. No adverse effects or toxicity were detected during treatment administration in any of the animals. To assess the latter, the clinical signs of the animals were monitored. Following TNBS rectal induction, rats developed a colonic inflammatory reaction. After microscopic observation of the different colon parts, 95% of rats present signs of inflammatory damage, with the rectum part being the more affected. This fact can be explained by the induction method used. Also, by difference of body weight before and postinduction treatment was demonstrated that there is no difference between groups ($p > 0.05$) and therefore there is homogeneity between the populations in study (Table 2).

Histological examination of the colon revealed by damage score, a marked inflammatory cell infiltrate, characterized by crypt abcess and diffuse lymphocyte infiltration with glandular destruction, some tissues damaged of intestinal wall, and very little damage of mucosal architecture and edemas.

It should be noted that the results of one of the rats belonging to group C were discarded for exceeding in excess the mean values of damage that had the rest of rats of that group. However, when we compared the four groups of animals in a global way (considering all the parameters together) by a scoring system reported by Hunter et al.,[18] but modified, any difference was found ($p > 0.05$). However, when performing an individualized analysis the only parameter that showed statistical differences was inflammatory cell infiltrate parameter, perhaps the more relevant parameter by their implication in EII. After applying the Student's t test, significant statistical significance was detected (Table 3).

There is a significant difference between group C and the rest of groups, showing a value of $p < 0.05$. This has shown that treatment C (alginate/pectin + oil + HT) is much more effective than the others to counteract damage in inflammatory bowel infiltration of the rectum.

Images of histological samples were taken under the microscope, and the inflammatory infiltrate suffered in each group could be observed. In the Control group it is observed that the inflammatory infiltration is high. Groups A and B, respectively show inflammatory infiltration in a small area of the sample. Image belonging to group C shows no signs of inflammatory infiltration (Figure 1).

Therefore, treatment with hydroxytyrosol reduced colon injury stimulating a regeneration of the glands.

4. DISCUSSION AND CONCLUSION

In the present study, we can appreciate that hydroxytyrosol exerts a protective effect in TNBS-induced colitis. Recently, the hydroxytyrosol has also been shown to protect against colitis induced by dextran sodium sulphate (DSS), a chronic model of inflammation.[19,20] We chose TNBS based on previous studies.[5,21,22] These studies have demonstrated that TNBS allows the time of acute phase production to be controlled, has a continuous distribution, and lasts longer than other existing models, such as acetic acid or sodium dextran sulfate (DSS). Taking into amount these results, in the present study we examined the effect of administration of hydroxytyrosol via rectal, in TNBS-induced colitis in rats.

The choice of studying the antioxidant HT, instead of any other oil, is based on several studies,[23,24] that demonstrate its anti-inflammatory effect. It is the antioxidant most known for its benefits, thanks to the number of studies available, but there is no study demonstrating its effect on IBD. We could say for the first time that it is significantly effective in IBD, decreasing intestinal inflammation.

The effect of hydroxytyrosol, the main phenol of the olive oil, on the inflammatory response is well established.[12,25,26] In addition, the effect of the olive oil and the pectin on the inflammation has also been extensively reviewed.[9,11,16,17,27] Previous studies reported that oral administration of olive oil and hydroxytyrosol have beneficial effect in colitis inflammation and pain induced by carrageenan, a neutrophil-mediated acute

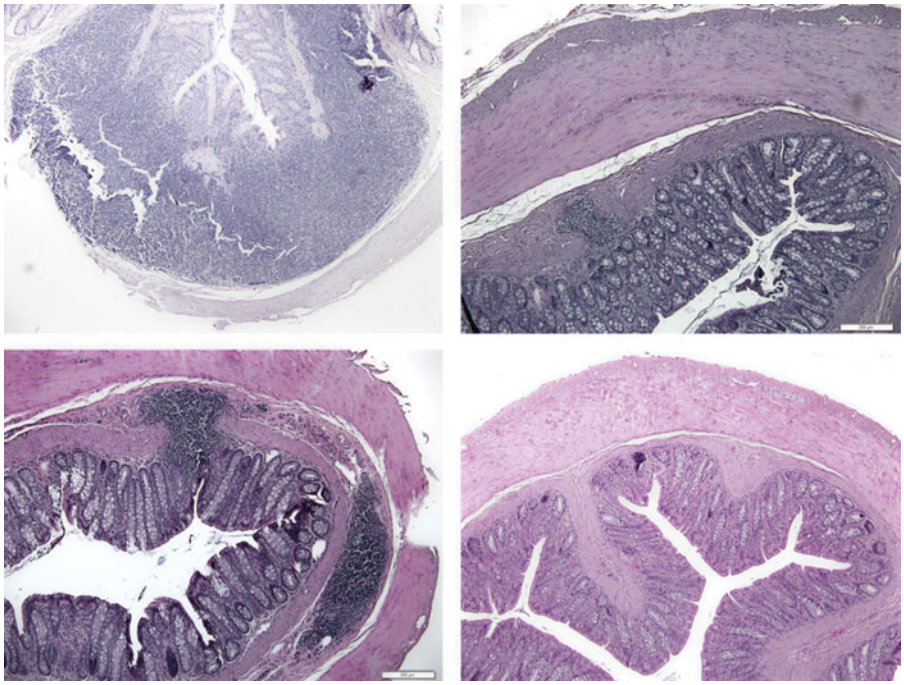

FIGURE 1 Hematoxylin and eosin histology of the rectum of the different groups. ×10 magnification. Upper left image: Control Group. Moderate inflammatory infiltration with involvement of the muscular layer of the wall, with damage to the mucosal architecture and without edema; Upper Right Image: Group A. Mild inflammatory infiltration. Muscle and mucosa layer involved. There is damage to the architecture of the mucosa and there is no edema; Lower left image: Group B. Mild inflammatory infiltration, with involvement of the mucosa, and damage to the mucosal architecture without edema; Lower Right image: Group C. Without histopathological alterations.

inflammatory response[20] or DSS,[28] a chronic model of this disease in rats. However, our experimental studies concerning the effect of hydroxytyrosol, olive oil, and pectin on TBNS-induced colitis would provide the first evidences that intrarectal administration of hydroxytyrosol and olive oil reduce the severity of the inflammatory damage of an acute type of colitis. In the present study, we have also observed that hydroxytyrosol has not had any secondary effect in the animal, nor presented any toxicity problem, in agreement with results obtained in other studies.[12,23,24,29]

In this project, the Hunter score was used to quantify the damage caused by the disease. There are other options, such as the use of biomarkers[30], but this involves manipulating and causing stress to the animal more than necessary to obtain the same results. We could say that the Hunter scale is valid for intestinal damage in IBD, as demonstrated by Hunter.[18]

The anti-inflammatory effect of hydroxytyrosol, as demonstrated in the present study, may be interesting for local treatment of colitis.

In the future, and after seeing that the HT helped in the treatment of the disease preclinically, the field of study could be broadened by looking for another type of emulsion that was richer in HT and to verify if a greater amount supposes greater effect beneficial, or if an excess has negative consequences.

It is recommended to continue the investigation by increasing the sample size, slightly modifying the treatments, and improving the procedure.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS

All applicable international, national, and/or institutional guidelines for the care and use of animals were

followed. The animals have been treated throughout the experimental process according to the Council of Europe agreements on the protection of animals used in animal experiments.

This article does not contain any studies with human participants performed by any of the authors.

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REFERENCES

1. Dignass A, Eliakim R, Magro F, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis Part 1: definitions and diagnosis. *J Crohns Colitis*. 2012;6:965–990. doi:10.1016/j.crohns.2012.09.003. PMID:23040452.
2. Van Assche G, Dignass A, Panes J, et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *J Crohns Colitis*. 2010;4:7–27. doi:10.1016/j.crohns.2009.12.003. PMID:21122488.
3. Zhang Y-Z, Li Y-Y. Inflammatory bowel disease: Pathogenesis. *World J Gastroentero*. 2014;20:91–99. doi:10.3748/wjg.v20.i1.91.
4. Reddy KVK, Naidu KA. Oleica cid, hydroxytyrosol and n-3 fatty acids collectively modulate colitis through reduction of oxidative stress and IL-8 synthesis; in vitro and in vivo studies. *Int Immunopharmacol*. 2016;35:29–42. doi:10.1016/j.intimp.2016.03.019. PMID:27016717.
5. Manjeshwhar SB, Nandhini J, Marikunte V, Arpit S, Venkatesh P, Raja F. Curcumin, an active component of turmeric in the prevention and treatment of ulcerative colitis: preclinical and clinical observations. *Food Funct*. 2012;3:1109–1117. doi:10.1039/c2fo30097d. PMID: 22833299.
6. Canadian Agency for Drugs and Technologies in Health. Golimumab (Simponi) (Subcutaneous Injection): Adult Patients with Moderately to Severely Active Ulcerative Colitis Who Have Had an Inadequate Response to, or Have Medical Contraindications for, Conventional Therapies [Internet]. Clinical Review Report. Ottawa ON: CADTH; 2014.
7. Sánchez-Fidalgo S, Cardeno A, Sánchez-Hidalgo M, et al. Dietary unsaponifiable fraction from extra virgin olive oil supplementation attenuates acute ulcerative colitis in mice. *Eur J Pharm Sci*. 2013;48:572–581. doi:10.1016/j.ejps.2012.12.004. PMID:23238173.
8. Takashima T, Sakata Y, Iwakiri R, et al. Feeding with olive oil attenuates inflammation in dextran sulfate sodium-induced colitis in rat. *J Nutr Biochem*. 2014;25:186–192. doi:10.1016/j.jnutbio.2013.10.005. PMID:24445043.
9. De la Puerta R, Martínez-Domínguez E, Ruiz-Gutiérrez V. Effect of minor components of virgin olive oil on topical antiinflammatory assays. *Z Naturforsch*. 2000;55:814–819.
10. Lyons CL, Finucanel OF, Murphy AM, et al. Monounsaturated fatty acids impede inflammation partially through activation of AMPK. *FASEB J*. 2016;30(Suppl. 1):[296.5].
11. Muto E, Dell'Agli M, Sangiovanni E, et al. Olive oil phenolic extract regulates interleukin-8 expression by transcriptional and posttranscriptional mechanisms in Cacao-2 cells. *Mol Nutr Food Res*. 2015;59:1217–1221. doi:10.1002/mnfr.201400800. PMID:25708117.
12. Auñon-Calles D, Canut L, Visioli F, et al. Hydrolyzed olive vegetation water in mice has anti-inflammatory activity. *J Nutr*. 2005;135:1475–1479. doi:10.1093/jn/135.6.1475. PMID:15930455.
13. Ciriminna R, Meneguzzo F, Fidalgo A, Ilharco L, Pagliaro M. Extraction, benefits and valorization of olive polyphenols. *Eur J Lipid Sci Tech*. 2016;118:503–511. doi:10.1002/ejlt.201500036.
14. EFSA NDA Panel. Scientific Opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J*. 2012;10:2848. doi:10.2903/j.efsa.2012.2848.
15. Pontoniere P, Martiradonna D. Inflammation and olive polyphenols. A perspective review of supporting literature. *Agro Food Ind Hi Tec*. 2012;23:69–71.
16. Markov PA, Popov SV, Nikitina IR, Ovodova RG, Ovodov YS. Anti-inflammatory activity of pectins and their galacturonan backbone. *Russ J Bioorg Chem+*. 2011;37:817–821. doi:10.1134/S1068162011070132.
17. Popov SV, Markov PA, Popova GY, Nikitina IR, Efimova L, Ovodov YS. Anti-inflammatory activity of low and high methoxylated citrus pectins. *Biomed Prev Nutr*. 2013;3:59–63. doi:10.1016/j.bionut.2012.10.008.
18. Hunter M, Wang A, Hirota L McKay D. Neutralizing Anti-IL-10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced colitis. *J Immunol*. 2005;174:7368–7375. doi:10.4049/jimmunol.174.11.7368. PMID:15905584.
19. Sánchez-Fidalgo S, Sánchez de Ibarguen L, Cardeno A, Alarcón de la Lastra C. Influence of extra virgin olive oil diet enriched with hydroxytyrosol in a chronic DSS colitis model. *Eur J Nutr*. 2012;51:497–506. doi:10.1007/s00394-011-0235-y. PMID:21874330.
20. Gong D, Geng C, Jiang L, Cao J, Yoshimura H, Zhong L. Effects of hydroxytyrosol-20 on carrageenan-induced acute inflammation and hyperalgesia in rats. *Phytother Res*. 2009;23:646–650. doi:10.1002/ptr.2686. PMID:19067390.
21. O'Malley D, Dinan TG, Cryan JF. Interleukin-6 modulates colonic transepithelial ion transport in the stress-sensitive Wistar Kyoto rat. *Front Pharmacol*. 2012;3:190. doi:10.3389/fphar.2012.00190.
22. Mañé Almero J. In vivo experimental models of inflammatory bowel disease and colorrectal cancer. *Nutr Hosp*. 2007;22(2):178–89. PMID:17416034.
23. Granados-Principal S, Quiles JL, Ramirez-Tortosa CL, Sanchez-Rovira P, Ramirez-Tortosa MC. Hydroxytyrosol: from laboratory investigations to future clinical trials. *Nutr Rev*. 2010;68:191–206. doi:10.1111/j.1753-4887.2010.00278.x. PMID:20416016.
24. D'Angelo S, Manna C, Migliardi V, et al. Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metab Dispos*. 2001;29:1492–1498. PMID:11602527.
25. Aparicio-Soto M, Sanchez-Fidalgo S, Gonzalez-Benjumea A, Maya I, Fernandez-Bolanos JG, Alarcon-de-la-Lastra C.

Naturally occurring hydroxytyrosol derivatives: hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol modulate inflammatory response in murine peritoneal macrophages. potential utility as new dietary supplements. *J Agr Food Chem*. 2015;63:836–846. doi:10.1021/jf503357s.

26. Aparicio-Soto M, Sanchez-Hidalgo M, Cardeno A, Gonzalez-Benjumea A, Fernandez-Bolaños JG, Alarcon-de-la-Lastra C. Dietary hydroxytyrosol and hydroxytyrosyl acetate supplementation prevent pristane-induced systemic lupus erythematous in mice. *J Funct Food*. 2017;29:84–92. doi:10.1016/j.jff.2016.12.001.
27. Cardeno A, Sanchez-Hidalgo M, Alarcon-de-la-Lastra C. An up-date of olive oil phenols in inflammation and cancer: Molecular mechanisms and clinical implications. *Curr Med Chem*. 2013;20:4758–4776. doi:10.2174/09298673113209990159. PMID:23834184.
28. Sanchez-Fidalgo S, Villegas I, Cardeno A, et al. Extra-virgin olive oil-enriched diet modulates DSS-colitis-associated colon carcinogenesis in mice. *Clin Nutr*. 2010;29:663–673. doi:10.1016/j.clnu.2010.03.003. PMID:20427102.
29. Soni MG, Burdock GA, Christian, Bitler MS, Crea R. Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. *Food Chem Toxicol*. 2006;44:903–915. doi:10.1016/j.fct.2006.01.008. PMID:16530907.
30. Coello C, Fisk M, Mohan D, et al. Quantitative analysis of dynamic 18F-FDG PET/CT for measurement of lung inflammation. *EJNMMI Res*. 2017;7(1):47. doi:10.1186/s13550-017-0291-2. PMID:28547129.

BLOQUE II

Título: Physical and functional properties of pectin-fish gelatin films containing the olive phenols hydroxytyrosol and 3,4-dihydroxyphenylglycol [1].

Autores: Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, Blanca Vioque, Fátima Rubio-Senent, Juan Fernández-Bolaños

Publicación: Carbohydrate Polymers

Título: Effect of edible pectin-fish gelatin films containing the olive antioxidants hydroxytyrosol and 3,4-dihydroxyphenylglycol on beef meat during refrigerated storage [2].

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Publicación: Meat Science

Resumen Bloque II

Según la FAO, actualmente un tercio de la producción mundial de alimentos se estropea o se desperdicia antes de ser consumido por las personas. El desperdicio o pérdida de estos alimentos puede ocurrir en cualquier punto de la cadena, desde el inicio en la propia producción hasta el punto final en los hogares.

Las enfermedades postcosecha de frutas y verduras son un problema importante en el almacenamiento de productos. En concreto la fresa es una fruta que se consume en todo el mundo presentando una vida media bastante corta, ya que se deteriora fácilmente debido a los daños mecánicos, la pérdida de agua y la aparición de hongos (*Botrytis Cinerea y Rhizopus Stolonger*) que son de suma importancia.

Mientras que la oxidación de los lípidos es el principal factor que contribuye al deterioro de la carne cruda durante el almacenamiento, causando una reducción de la calidad y aceptabilidad debido al desarrollo de un sabor y rancidez indeseables, es por ello que se hace necesario el uso de antioxidantes. Sin embargo, debido al riesgo potencial para la salud de algunos antioxidantes sintéticos comúnmente utilizados en la industria alimentaria, como el butil hidroxianisol (BHA) o el butil hidroxitolueno (BHT), es conveniente reemplazar estos antioxidantes convencionales con productos antioxidantes naturales.

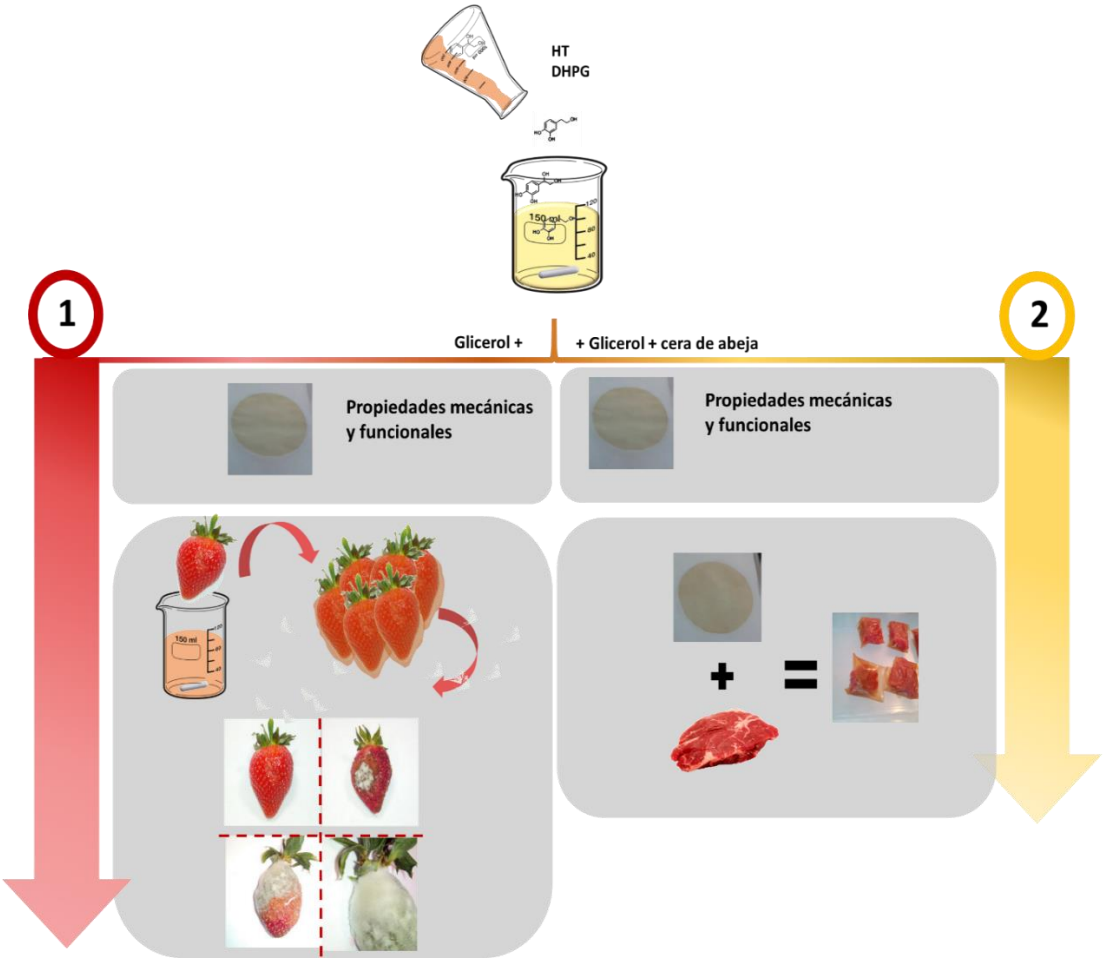
El recubrimiento comestible o película comestible es una alternativa interesante para mejorar la conservación de los alimentos ya que permite controlar los procesos, intercambio de gases, el transporte de compuestos antimicrobianos, así como de compuestos antioxidantes, lo que permitirían prolongar la vida útil del producto.

En este estudio se ha realizado un recubrimiento y película comestible mediante la formulación con un carbohidrato (pectina cítrica) y de una proteína de pescado (gelatina). Además, un nuevo enfoque para el recubrimiento/película comestible fue agregar dos

fenoles naturales con propiedades antioxidantes y antimicrobianas, hidroxitirosol (HT) y 3,4-dihidrofenilglicol (DHPG) presentes en la aceituna.

El primer objetivo de este estudio fue caracterizar dos recubrimientos comestibles en base de gelatina de pescado y pectina cítrica los cuales presentan diferente proporción de un plastificante, en este caso glicerol. Además, se le agregaron los antioxidantes y antimicrobianos, el HT y el DHFG, para su posterior aplicación en fresas y su posible efecto antifúngico. Los resultados mostraron que aquellos recubrimientos que presentaban HT/DHPG en su formulación retrasaron significativamente la aparición de moho en las fresas [1].

Para el caso de la carne cruda el objetivo del estudio ha sido evaluar el efecto de la adición de HT y DHPG, a una película comestible de gelatina de pescado y pectina sobre la preservación de la carne de ternera cruda, durante el almacenamiento a 4 ˚C. También se preparó una nueva película compuesta que incluía cera de abejas, lo que resultó en una reducción de la permeabilidad al oxígeno de la película. Los resultados experimentales mostraron que las muestras de carne envueltas con una película que contenía antioxidantes redujeron la formación de productos de oxidación en forma de sustancias de reacción del ácido tiobarbitúrico (TBARS) en comparación con la película de control sin antioxidantes.



Esquema resumen bloque II

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Physical and functional properties of pectin-fish gelatin films containing the olive phenols hydroxytyrosol and 3,4-dihydroxyphenylglycol

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ABSTRACT

This study describes the development of a composite edible film based on pectin and fish skin protein capable of protecting food from microbial attack and oxidative degradation. The film was prepared with glycerol as plasticizer and the antioxidant and antimicrobial phenolic compounds hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), extracted from olive fruit, as active agents. The influence of the concentration of plasticizer and active HT/DHPG on the mechanical and functional properties of the films was investigated, with values of water vapor permeability (WVP) between 0.13–0.22 g mm/h m^2 kPa and oxygen permeability (OP) between 9.91–40.76 cm^3 μm/m^2 d kPa. The release behavior in water at different pH values was also evaluated. The antimicrobial capacity of the novel food coating was tested on strawberries, a fruit with high perishability. The bioactive edible film containing HT/DHPG preserved the strawberries against mold during storage with a significant delay in visible decay.

1. Introduction

Pectin is a plant cell wall polysaccharide rich in d-galacturonic acid, which is mainly obtained from food processing industry waste (citric or apple peels). One application of pectin in the food industry is for film production, where films are used to improve the sensory attributes of certain foods and prolong their shelf-life (Khalifa, Barakat, E-Mansy, & Soliman, 2016). Pectin films have relatively good hardness and adhesiveness, with mucoadhesive and bioadhesive characteristics, and act as a good oxygen barrier. However, they present some limitations such as rigidity and brittleness, as well as high water sensitivity, thus pectin films act as a poor barrier for water (Farris et al., 2009). Therefore, some drawbacks in the mechanical properties of pectin film barriers must be improved.

Blending pectin with polymers, proteins, or other polysaccharides – via ionic linking, hydrogen bonding or hydrophobic interactions – may improve the characteristics of pectin-based materials for food applications, drug delivery, or tissue engineering (Barkay-Olami & Zilberman, 2016; Liu, Kost, Yan, & Spiro, 2012). Pectin-protein blended films offer advantages in terms of mechanical properties and water-vapor permeability (WVP) with respect to the films formed from pectin or protein alone (Di Pierro et al., 2013). Concretely, the development of composite films formed from ionic interactions between pectin and gelatin at different polymers ratios was reported by Farris et al. (2009). The final film characteristics depended on the specific combination of materials and conditions, including the source and type of pectin and gelatin, pH of the reaction mixture, and the pectin-gelatin ratio. High gelatin concentrations yielded elastic and stretchable films, whereas high pectin proportions made the films stiffer and more brittle. In this work, we developed a citrus pectin (high-methoxyl pectin 53%)-fish skin gelatin (ratio 1:1) blend film with improved properties. Previously developed films with similar components but different proportions were considered to have potential applications as packaging or coating material for the food or drug industries (Liu, Fishman, & Hicks, 2007).

In addition, we incorporated two phenolic antioxidants naturally found in olive fruit, hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), to add antimicrobial functionality to the film material. The antifungal activity of HT has been reported (Yangui, Dhouib, Rhouma, & Sayadi 2009; Yangui, Sayadi, Rhouma, & Dhouib, 2010), whereas the antifungal effect of DHPG has not yet been studied. HT also possesses anti-carcinogenic and anti-inflammatory properties, and presents a broad range of beneficial physiological activities on plasma lipoprotein, oxidative damage, platelet and cellular function, as well as on bone health (Hu, He, Jiang, & Xu, 2014; Killeen,

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Pontoniere, & Crea, 2011). The recognition of HT's impressive health benefits has led to its use as a dietary supplement and functional food. It has been granted GRAS (generally recognized as safe). In contrast, few studies have been conducted until now on the functional properties of DHPG, structurally similar to HT and with an additional hydroxyl group in the β-position, because it has only recently been isolated (De Roos et al., 2011; Rodríguez-Gutiérrez et al., 2012). To the best of our knowledge, the use of these phenolic compounds as antioxidant and antimicrobial components of edible films has not previously been reported.

2. Hypotheses

The development of a pectin-gelatin film blended with the active, natural olive phenolic compounds HT and DHPG would have a wide range of potential applications. In particular, as a food coating or packaging, the HT/DHPG films would help control spoilage and the proliferation of pathogens in food during its storage, to improve the shelf life and quality of food. Additionally, edible HT/DHPG films would impart potential health benefits when consumed and the phenolic compounds released. These bioactive films may also be of interest for biomedical and pharmaceutical applications (controlled drug delivery system, tissues engineering, organ regeneration, and bioadhesive for wound dressing) (Farris et al., 2011). As an example, an alginate bilayer film containing HT was recently developed for potential use as a topical chemotherapeutic agent for skin and breast cancer treatment (Ng & Tan, 2015) or as an alternative treatment for rheumatoid arthritis due to their ant-inflammatory activity (Ng, Tan, & Buang, 2017).

The aim of this study was to analyze the effect of the olive phenolic compounds HT and DHPG on the structural, mechanical, and functional properties of pectin-gelatin blend films, as well as their dissolution behavior. In addition, the antifungal effect of the film, with and without the antimicrobials, was evaluated on strawberries, a fruit with high perishability.

3. Materials and methods

3.1. Materials

HT and DHPG were extracted and purified from olive by-products by a chromatographic system based on ion exchange, as described by Fernández-Bolaños et al. (2011) and Fernández-Bolaños et al. (2014). Citrus pectin (galacturonic acid content, 91%; degree of methyl esterification, 53%), gelatin from fish skin (~95% purity), and glutaraldehyde solution 25% in H_2O were purchased from Sigma-Aldrich (St Louis, MO, USA). Glycerol was purchased from Panreac Química, S.A. (Barcelona, Spain).

3.2. Preparation of the film

Films were prepared by dissolving 0.5 g of citrus pectin and 0.5 g of fish gelatin in 20 ml of distilled water with continuous stirring at room temperature for 2 h. The pH of the mixture was adjusted to 8 using 4 M NaOH. Then, the pH was gradually reduced to 3–4 with 1 M HCL. Glutaraldehyde (50 μl/g polymer) and glycerol at two concentrations (1 g/g polymer, Film 1, or 0.5 g/g polymer, Film 1/2) as a plasticizer were added to the solution, and the mixture was stirred vigorously for 30 min. After complete dispersion, the phenolic compounds HT and DHPG were added to the mixture with a concentration range of HT 0.03–1.8 mg/mL and DHPG 0.03–0.18 mg/mL. The resultant gel was degassed and spread on a polypropylene film followed by drying for 48 h at room temperature. The same methodology was used to prepare films containing no phenolic compounds (film control, without the addition of HT and DHPG), or the coating for strawberry fruits without addition of glutaraldehyde. All experiments were carried out at room temperature.

3.3. Film thickness

Film thickness, expressed in μm, was measured using a micrometer (Baxlo 4000/Film) with an accuracy of 0.001 mm. Measurements were taken at five random sites on each film and for five films of each formulation. The mean and standard deviation were calculated for the evaluation of mechanical properties.

3.4. Mechanical behavior

The mechanical properties were measured using a 3340 Series Single Column System Instron Instrument, model LR30 K (Fareham Hants, UK), equipped with a load cell 2 kN and following the ASTM standard method D882 (ASTM, 2002). Films were cut into strips of 100 × 10 mm and mounted between the tensile grips of the instrument and stabilized at 25 °C and 50% relative humidity. With an initial grip separation of 60 mm, the samples were stretched at a speed of 10 mm/min until breakage. The average of five measurements was taken. The force-distance data obtained in the test were transformed into stress-strain curves and the mechanical parameters were calculated as elastic modulus (E), tensile strength at break (TS), and elongation at break (EB). E and TS were expressed as kPa and EB as percentage.

3.5. Oxygen permeability

The oxygen permeability (OP) of the films was analyzed using an oxygen transmission rate system (Mocom, USA) following the standard method (ASTM, 2014). An effective area of 50 cm^2 was exposed to permeation in test conditions of 25 °C and 50% relative humidity. Assays were performed in triplicate and the mean values were expressed as cm^3 μm/m^2 d kPa.

3.6. Water vapor permeability

Water vapor permeability (WVP) was determined following the gravimetric method ASTM (2016). Film samples were sealed over a circular opening of an aluminum permeation container filled with anhydrous $CaCl_2$. The cells were kept in a hermetically closed chamber containing an oversaturated solution of Mg $(NO_3)_2$ at 22–25 °C in order to maintain a relative humidity difference of 95%. The WVP was determined from the slope obtained for the regression analysis of weight loss data versus time, once the steady state had been reached, divided by the film area. Results were expressed as g mm/h m^2 kPa. All tests were carried out at least in triplicate and an empty aluminum cup covered with the film was used as control.

3.7. Solubility of film components and bioactive compounds

All films were pre-dried under desiccator for 48 h prior to testing. The solubility of the composite films was tested in water at different pH values. Specimens (4 × 2 cm) were placed in 50 ml of 0.1 M acetate (pH 4.0), phosphate (pH 7.2), or Tris-HCl (pH 8.5) buffer solutions for 1, 24 and 48 h. The rest of the specimens were removed, and the amounts of pectin and protein from the films that had solubilized into the different solutions were measured by uronic acids assay (Blumenkrantz & Asboe-Hansen, 1973) and Bradford assay (Bradford, 1976), respectively. The HT/DHPG released was determined by HPLC according to a previously published method (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012).

3.8. Color

Film color was assessed by the measurement of tristimulus color coordinates in the CIELAB scale with a spectrocolorimeter (BYK-Gardner, USA). The color was expressed in terms of lightness or brightness (L*) and the chromaticity parameters as redness or greenness

(a*) and yellowness or blueness (b*). These values were then used to calculate the hue degree (h° = arctan (b*/a*)), as well as chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$), which indicated the intensity or color saturation. The colorimeter was warmed up for 30 min and calibrated with a white standard. Film measurements were conducted at least 5 times. The skin color of strawberries was determined on five strawberries in four different locations.

3.9. Electron microscopy (SEM)

Cryoscanning electron microscopy (Cryo-SEM) was used to examine microstructural representative cross sections of edible coating. Samples were frozen in liquid nitrogen for 2 min and then transferred to the preparation unit. Edible coatings were fractured under cryogenic conditions. After ice sublimation, the fragments were coated with a thin layer of gold and palladium using a Leica AC600, and transferred into the cold stage of the SEM chamber. Specimens were observed with a Zeiss EVO SEM microscope (Zeiss, Oberkochen, Germany) at −135 °C under a 5 kV acceleration potential.

3.10. Strawberries collection

Strawberries were purchased from a local market during the months of April–May. Strawberries homogenous in size, color, and appearance, without signs of mechanical damage and fungal infection, were manually selected for treatment.

3.11. Application of films on strawberries

The film coating was made by immersing the strawberries in the mixture pectin-protein-glycerol, prepared as described in Section 3.2. The antimicrobial agents HT and DHPG were added to the coating solution and incorporated into the blend by mixing with magnetic stirring for 5 min. Fruits were randomly assigned into three groups of 10 strawberries: an uncoated group with no manipulation, a coated group used as film control, and a coated group with HT and/or DHPG. Strawberries were immersed in the mixture for 1 min, the excess film-forming solution was drained, and the coated strawberries were dried in a chamber with forced air (Fig. 1a). The strawberries were then placed on a perforated plastic mesh and stored at room temperature for several days (Fig. 1b). All assays were conducted at room temperature.

3.12. Visual decay: punctuation

The appearance of mold spots on the strawberry samples was evaluated by sensory inspection by ten trained panelists. Each panelist inspected all groups of strawberries without knowing what treatment corresponded to each group. Fruits were considered infected when a visible contamination, characterized as brown spots and the presence of mycelium of molds on the fruit surface, was observed. The results were expressed as fruit infection percentage by measuring the area of mold growth, according to the next classification: 0–1, complete absence of spots; 1–2, the presence of less than 50% of mold, and 2–3, the presence of more than 50% of mold (Fig. 1c–f).

3.13. Statistical analysis

Results were expressed as mean values ± standard deviations. STATGRAPHICS® plus software was used for statistical analysis. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) and the LSD method. A p-value < 0.05 was considered significant.

4. Results and discussion

We investigated the properties of an edible active film produced from an aqueous mixture of protein, a fish skin gelatin, and the polysaccharide pectin (ratio 1:1) plasticized with glycerol. The natural antioxidant and antimicrobial compounds from olives, HT and DHPG were also incorporated. The films require flexibility to avoid breakage during their preparation and use. Two concentrations of glycerol were added to the formulation (1 g/g polymer for Film 1, and 0.5 g/g polymer for Film 1/2) and the main mechanical and functional properties of the obtained films (permeability to oxygen and water vapor) were assessed, as well as their adhesion to the fruit and solubility.

The three pectin-fish skin gelatin composite films – plasticized with

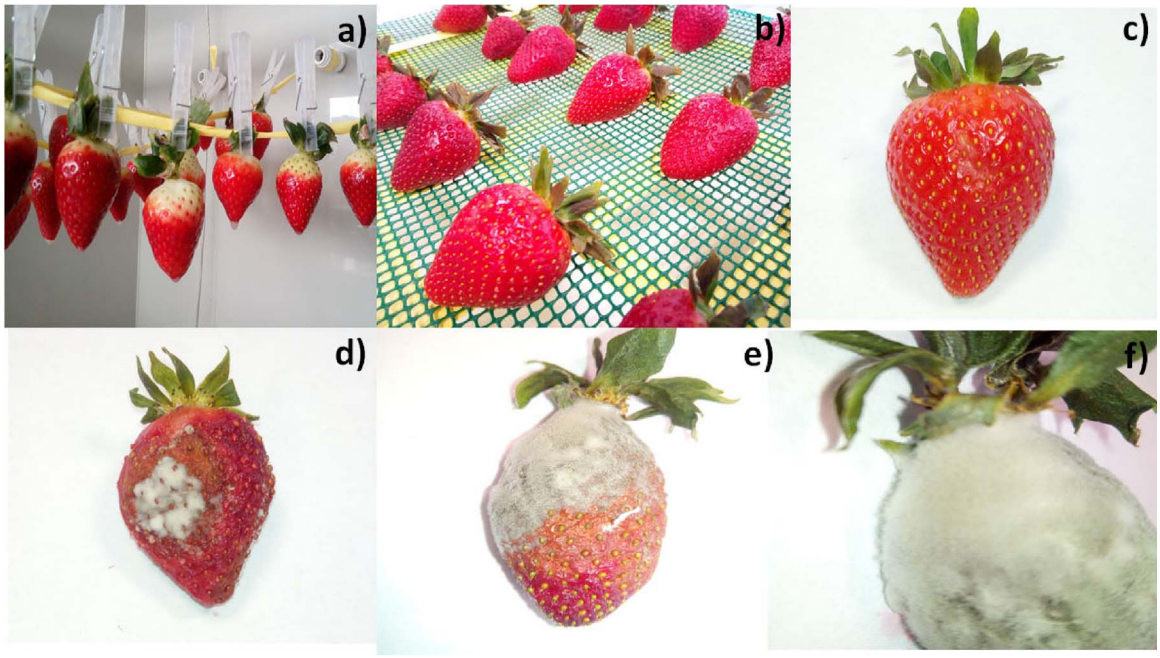

Fig. 1. a) Drying of the strawberries after their immersion in the edible coating solution. b) Storage of the strawberries during the test; uncoated strawberries served as controls. c) A strawberry with 0% mold infection. d) A strawberry with approximately 25% mold infection. e) A strawberry with approximately 50% mold infection. f) A strawberry with 100% mold infection.

Table 1
Mechanical and functional properties of pectin-fish skin gelatin composite films plasticized with glycerol at two concentrations (Film 1 and Film 1/2) and film with bioactive compounds (BC) added. Young's elastic modulus (E), Tensile strength (TS), Elongation at break (EB), Oxygen Permeability (OP), and Water vapor permeability (WVP). Mean values ± standard deviation.

	Thickness (μm)	E × 10^3 (kPa)	TS × 10^3 (kPa)	EB (%)	OP (cm^3 μm/m^2 d kPa)	WVP (g mm/h m^2 kPa)
Film 1	151.50 ± 0.00	123.51 ± 16.10*			40.76 ± 5.32*	0.22 ± 0.02*
Film 1/2	120.26 ± 0.46	205.43 ± 34.73	9.54 ± 1.15	41.80 ± 5.50	9.91 ± 4.36	0.16 ± 0.01
Film 1/2 with BC	120.86 ± 3.46	287.62 ± 54.51#	16.06 ± 1.48#	53.40 ± 7.83#	11.23 ± 0.60	0.13 ± 0.04

* Significant difference ($p < 0.05$) between formulations with different amounts of plasticizer.
\# Significant difference ($p < 0.05$) between formulations with and without BC.

glycerol at the higher concentration (Film 1), at half the concentration (Film 1/2), and blended with bioactive compounds (Film 1/2 with BC) – had a satisfactory soft texture and stickiness; they were flexible and easy to peel from the polypropylene film. In this study, a tensile test was performed to evaluate the influence of the addition of a combination of the active phenols HT and DHPG (0.06 + 0.06 mg/mL of mixture) on the mechanical properties of the pectin-protein composite film.

4.1. Film mechanical properties

Young's elastic modulus E (kPa), tensile strength TS (kPa), and elongation at break EB (%) were determined for films with HT/DHPG and compared with the HT/DHPG-free films (Film 1/2) (Table 1). The incorporation of phenolic compounds caused a significant increase of 1.7-fold ($p < 0.05$) in the tensile strength of the films. The control film's percent elongation at break was 42%, which increased to 53% for the film containing HT/DHPG, representing a significant difference ($p < 0.05$) in the stretching capacity of the bioactive film. The hydroxyl groups of the phenolic compounds could interact with the polymer side chains of pectin and/or protein to make the film slightly stronger and more stretchable. A similar effect was described due to electrostatic interactions between the hydroxyl groups of carvacrol with caseinate films (Arrieta, Peltzer, Garrigós & Jiménez, 2013). Also, the use of different phenolic compounds to strengthen various protein-based films via protein-polyphenol interactions, with hydrogen bonds between the hydroxyl groups of phenols and carbonyl groups of protein molecules, has been reported (Prodpran, Benjakul, & Phatcharat, 2012). Conversely, film preparation with HT/DHPG caused a slight reduction in film elasticity, with a slightly higher but significant value of Young's modulus ($p < 0.05$) probably because the phenolic compounds could form hydrogen bonds with the polymer chains, modifying the network of film and leading to an increased elastic modulus.

The influence of glycerol addition to the film and its effect on the Young's elastic modulus was also studied. Increasing the amount of plasticizer improved the film's flexibility, with a fall in elastic modulus from (205.4 ± 34.7) × 10^3 kPa for Film 1/2 to (123.5 ± 16.1) × 10^3 kPa for Film 1 with double the amount of glycerol added. Plasticizers, such as glycerol, are able to disperse between polymer chain spaces, reducing the intermolecular attraction between polymer chains and increasing the flexibility of the film (Ng & Tan, 2015).

4.2. Film permeability

The bioactive films, with HT and DHPG incorporated, were submitted to oxygen and water barrier assays and the results compared to the values obtained for HT/DHPG-free film (Film 1/2) (Table 1). The pectin-gelatin composite films plasticized with low glycerol (Film 1/2) had an adequate low permeability to oxygen (OP) and water vapor (WVP), which were not affected by the incorporation of HT and DHPG, without significant differences. Rojas-Graü et al. (2006) developed an edible apple puree film that showed a good OP with a value of 22.6 cm^3 μm/m^2 d kPa, 2-fold higher than the value obtained in the present work. Porta et al. (2016) reported an OP value of 76 cm^3 μm/m^2 d kPa for a blended film of pectin and bitter vetch protein, and therefore worse than in our case. The cross-linking of polymer pectin-protein caused a decrease in film permeability, with an OP value even lower than polyethylene film, at 60.1 cm^3 μm/m^2 d kPa (Di Pierro, Mariniello, Giosafatto, Masi, & Porta, 2005).

The barrier effect to water or WVP, is not affected by the protein-pectin crosslink formation but instead depends mainly on the hydrophilicity of the film (Farris et al., 2011). In our case the values of WVP (Table 1) were similar to the film developed with pectin and fruit extracts, 0.104–0.160 g mm/m^2 h kPa (Eça, Machado, Hubinger, & Menegalli, 2015), and slightly lower than the film developed with pectin and bitter vetch protein, 0.67 g mm/m^2 h kPa (Porta et al., 2016), or with skin fish gelatin, 0.21 g mm/m^2 h kPa (Nuñez-Flores et al., 2013). However, a slight but significant increase in OP and WVP ($p < 0.05$) was obtained for the films with a higher amount of plasticizer. This result is in agreement with previous studies, where the plasticizer reduced intermolecular forces between the polymers chains, leading to a less dense polymer network and increased free volume, with the consequence that films were more permeable to oxygen and water molecules (Cuq, Gontard, Cuq, & Guilbert, 1997).

4.3. Film color

The effect of the incorporation of HT/DHPG, at a concentration of

Table 2
Color parameters (L^*, a^*, b^*) and chroma C^* and hue ho values of the pectin-fish skin gelatin composite film with bioactive compounds (BC) and without BC (control). Control parameter of uncoated and coated strawberries with and without bioactive compounds (BC).

	L^*	a^*	b^*	C^*	h^o
Film control	19.93 ± 2.30	−1.64 ± 0.11	3.17 ± 0.72	3.58 ± 0.62	118.29 ± 6.35
Film with BC	24.78 ± 6.23	−1.32 ± 0.49	5.48 ± 2.41	5.66 ± 2.39	104.50 ± 6.64*
Uncoated strawberries	30.57 ± 2.12	24.67 ± 1.52	16.02 ± 3.04	29.50 ± 2.21	32.83 ± 4.97
Coated strawberries (control)	30.50 ± 2.17	24.27 ± 3.99	15.15 ± 3.40	26.82 ± 6.63	31.79 ± 3.90
Coated strawberries with BC	28.14 ± 3.41	22.54 ± 1.84	14.29 ± 3.40	26.80 ± 2.72	32.10 ± 6.07

* Significant difference ($p < 0.05$) between formulations with and without BC.

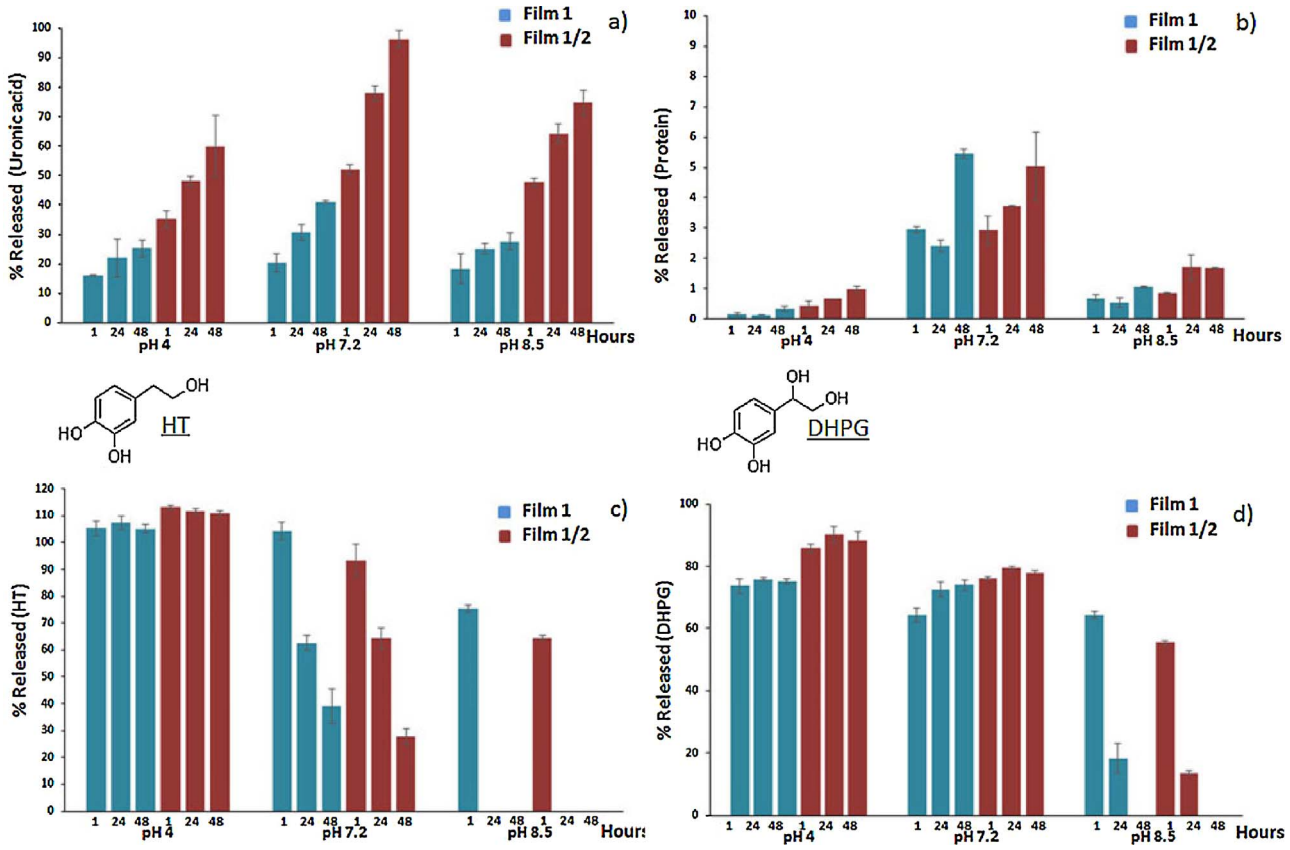

Fig. 2. Release (%) of pectin (a), protein (b), hydroxytyrosol (HT) (c), and 3,4-dihydroxyphenylglycol (DHPG) (d), from pectin-skin fish protein composite film containing two concentrations of glycerol added at different pHs (4, 7.2, and 8.5) after 1, 24, and 48 h incubation at room temperature. The results are expressed as the mean values and the bars indicate standard deviation.

0.06 mg/mL, on the color properties of the pectin-protein film was evaluated using L*, a*, and b* factors (Table 2). The incorporation of bioactive compounds had no significant effect on the color factors. However, the film with bioactive compounds was slightly brighter (higher mean L* value), and had a slightly yellower tone (higher b* values) than the control film. The hue angle (h^o) was the only parameter whose value significantly decreased ($p < 0.05$), indicative of the tendency for browning of the film blended with phenolic compounds at the concentration assayed.

4.4. Solubility of film components and bioactive compounds

The water solubility of the edible films prepared with HT or DHPG was investigated after 1, 24, and 48 h of incubation at different pHs. Electrostatic interaction between oppositely-charged biopolymers produce polyion complexes with improved performances compared to networks of individual polymers. Therefore, pH is the most important and most widely used factor for controlling the strength and characteristic of the hydrogel formed (Farris et al., 2009). The release of pectin, protein, HT and DHPG was measured (Fig. 2). A lower solubility of pectin and protein was observed at pH 4 for the films with the two glycerol concentrations assayed (Fig. 2a and b). This is in agreement with the result of Liu et al. (2007), who reported the importance of pectin pKa (3.55–4.10) and protein pI (4.8–5.2) to establish the formation of an electrostatic complex. At pH 4.0, electrostatic complexes between the carboxyl group of pectin and the ammonia group of the polypeptide chains can be formed, stabilizing the film network and reducing its solubility. For this reason, at pH 4 where the net charge is zero next pKa of pectin and pI of protein, interaction with water in minimized while polymer interactions are maximized yielding highly structured coacervate networks (Farris et al., 2009). A marked decrease in the solubility of pectin was observed at different pHs for the film with half the amount of glycerol added. For Film 1/2, almost 100% of uronic acid was released at pH 7.2 following 48 h incubation, while Film 1 (with double the amount of glycerol) only reached about 38% of uronic acid released (Fig. 2a). However, a significant decrease was observed in the release of solubilized protein and there were no significant differences between the films plasticized with more (Film 1) or less (Film 1/2) glycerol (Fig. 2b).

The release of HT and DHPG from the films obtained with two concentrations of glycerol was also evaluated at different times and pHs (Fig. 2c and d). Initially the two film formulations showed a complete release of HT at 1 h at pH 4.0 and pH 7.2, whereas 76% and 90% DHPG was released after 24 h at pH 4 for Film 1 and Film 1/2, respectively, compared to 73% and 80% at pH 7.2. However, while HT was stable at pH 4.0 during 24 and 48 h, the phenolic compound was degraded at pH 7.2 after 24 h of storage. For pH 8.2, an important amount of HT and almost all of the released DHPG was degraded after 24 h and 48 h incubation.

Therefore, HT and DHPG were homogeneously incorporated into pectin-protein polymers and rapidly released, with the total release of HT and a slightly lower release of DHPG. DHPG contains an additional hydroxyl group compared to HT, which might form a stronger complex with the pectin by hydrogen bonding, as described by Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, and Fernández-Bolaños (2017).

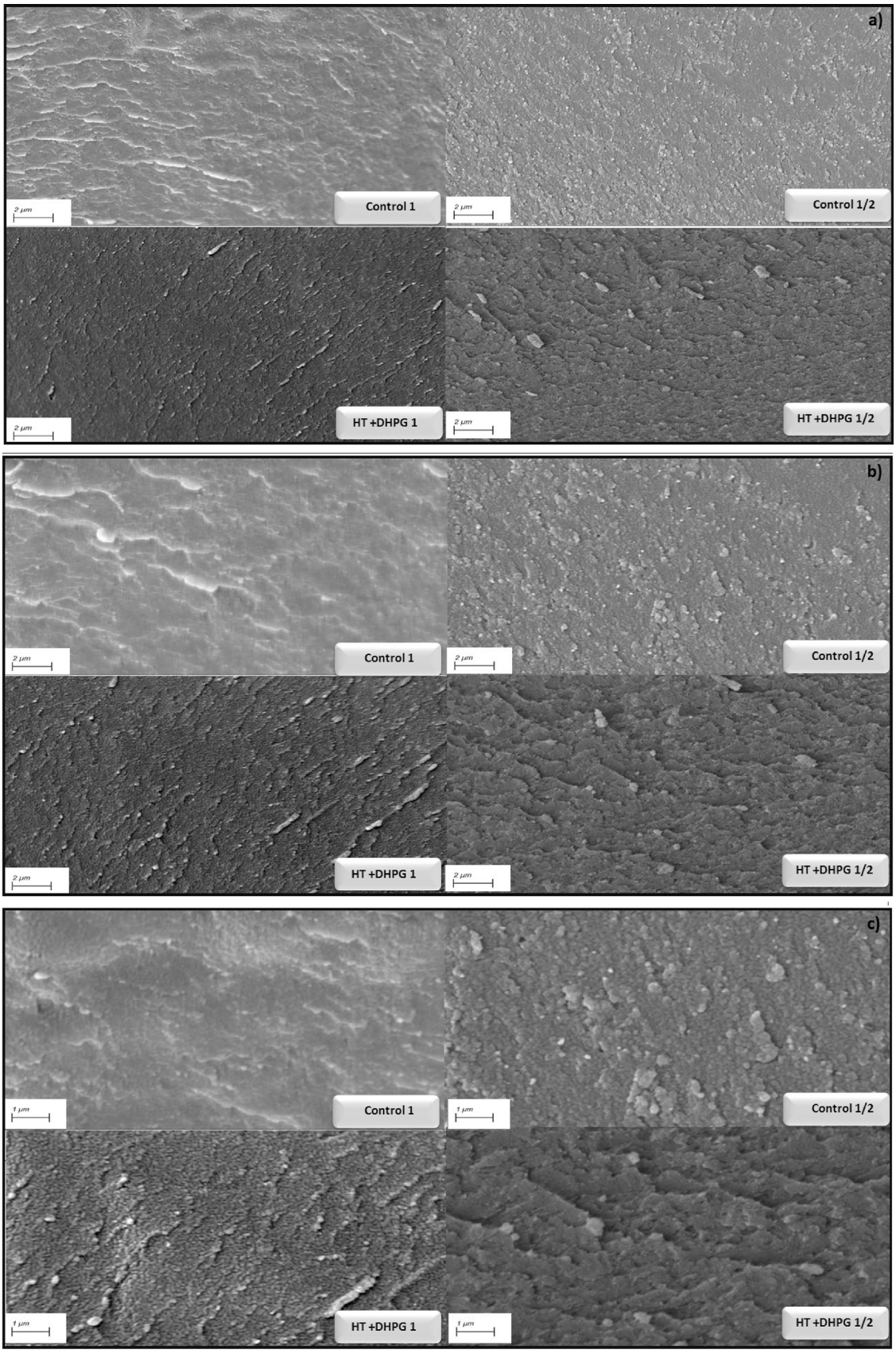

Fig. 3. Cryo-SEM micrographs of cross-sections of the pectin-fish skin protein films containing two concentrations of glycerol (Film 1, Film 1/2) and in the presence or absence of bioactive compounds (HT plus DHPG) at three different magnifications a) 2500x, b) 5000x, and c) 10,000x.

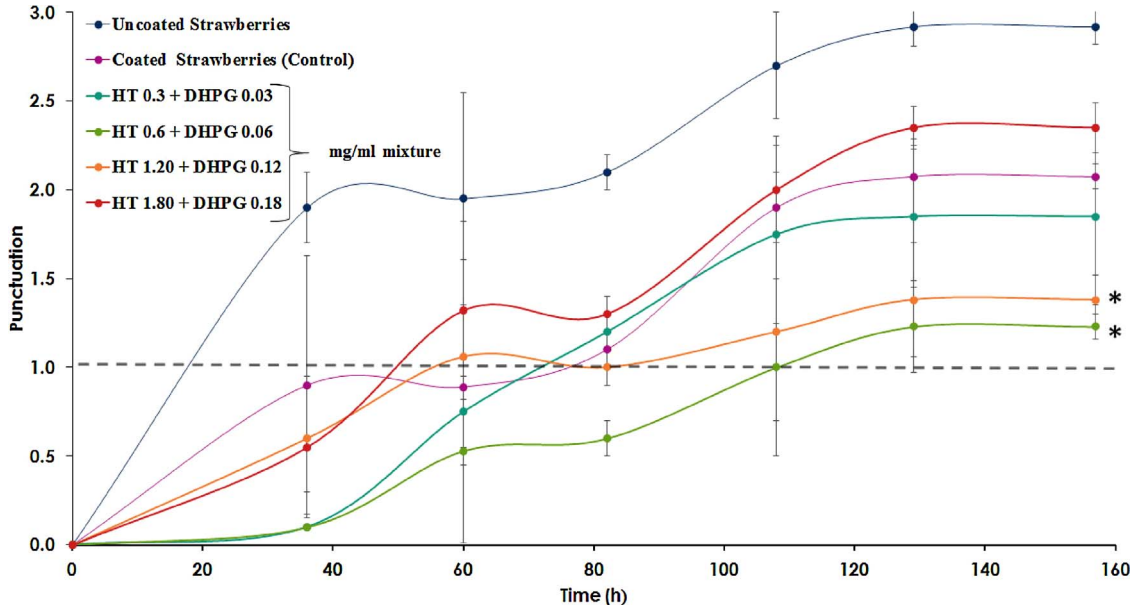

Fig. 4. Average punctuation of decay for strawberries covered with an edible pectin-fish skin protein film-forming formulation containing a mixture of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) at different concentrations in the ratio HT: DHPG (10:1) and glycerol 0.5 g/g polymer, during storage at room temperature for 157 h. Comparison with uncoated strawberries and with coated strawberries without bioactive compounds (control). Dotted line show the average punctuation that indicate the begining of the presence of mycelium of molds in a wide number of strawberries* Significant difference at 157 h compared to the uncoated strawberries and the control ($p < 0.05$).

4.5. Scanning electron microscopy (SEM) of the films

Differences in film morphology due to the addition of glycerol and HT/DHPG were investigated by scanning electron microscopy (SEM) (Fig. 3). Micrographs of the edible film surfaces without HT/DHPG exhibited a well-organized homogeneous structure and the film with a higher concentration of glycerol had a smoother film surface. Cryo-SEM revealed that the incorporation of HT/DHPG led to a film with a featureless and nonporous morphology, indicating a good dispersion of the bioactive compounds, although the addition of HT/DHPG caused a slight change to the film surface with a certain disruption of the smooth and homogeneous structure.

4.6. Effect of HT/DHPG in pectin-protein film on visible decay of strawberries

Strawberries, a highly perishable fruit, especially susceptible to decay, was used as a model to evaluate the ability of the bioactive edible coating containing natural antioxidant and antimicrobial agents to preserve the shelf life of foods.

The pectin-protein-based films containing HT/DHPG were evaluated for their effectiveness as an antimicrobial coating against mold growth on strawberries. In the first experiment, the bioactive compounds HT/DHPG were tested in the ratio of 10:1, the proportion naturally found in olives, and the change in visible decay during storage at room temperature was evaluated (Fig. 4). The strawberries coated with the pectin-protein film-forming solution presented a significantly ($p < 0.05$) lower amount of micelle growth compared to the uncoated strawberries after 157 h of storage. The mold reduction was even more evident for the strawberries that were coated with a mixture of HT: DHPG (10:1).

Indeed, while the uncoated strawberries started to show clear signs of mold growth after 36 h (with an average punctuation of 1.9), strawberries coated with HT: DHPG film did not develop clear fungal decay (with an average punctuation above 1) until 82 h of storage for 1.2 mg HT/mL: 0.12 mg DHPG/mL, or until 108 h for the concentration of 0.6 mg HT/mL: 0.06 mg DHPG/mL. In addition, strawberries coated with these two film formulations showed a significant difference ($p < 0.05$) at the end of storage at 157 h, compared to uncoated strawberries and the film control (without the antimicrobial phenolic compounds), as well as when compared to the other two HT: DHPG concentrations tested.

Further experiments were carried out to assess the bioactive compounds individually. The bioactive films containing 0.06 mg of DHPG/mL (Fig. 5a) and 0.06 mg of HT/mL (Fig. 5b) were found to be the most efficient for each of different experiments carried out at different times and different concentrations, showing a significant difference ($p < 0.05$) with respect to the film control and uncoated strawberries at the end of storage at 157 h. Again, strawberries coated with the film control presented a significantly lower amount of mold growth than uncoated strawberries ($p < 0.05$). All coated fruits, with and without bioactive compounds, showed much lower disease during the storage period, demonstrating the ability of the pectin-protein film coating to protect strawberries from microbial attack and prolong their shelf-life. The incorporation of HT or DHPG into the edible film improved the postharvest quality of the strawberries. Differences between the antimicrobial effects of HT and DHPG on the preservation of the fruit indicate that the agents respond differently according to the type of fungi or the ripeness of the fruit. However, further research is required to improve the antifungal activity of each compound against the most common strains of molds responsible for strawberry decay.

A last experiment was carried out to evaluate the influence of the concentration of plasticizer added to the edible coating formulation on strawberry decay during six days of storage at room temperature, with and without the incorporation of the antimicrobial agents, at the most effective concentrations found in previous assays (Fig. 6). A better preservation of strawberries was observed for the film with a higher concentration of plasticizers, although the strawberries coated with Film 1/2 also showed significant difference ($p < 0.05$) with respect to uncoated strawberries. Films containing the antimicrobial agents HT: DHPG at 0.06 mg/mL each were the most efficient, with the lowest (statistically significant) fungal decay indexes observed for the

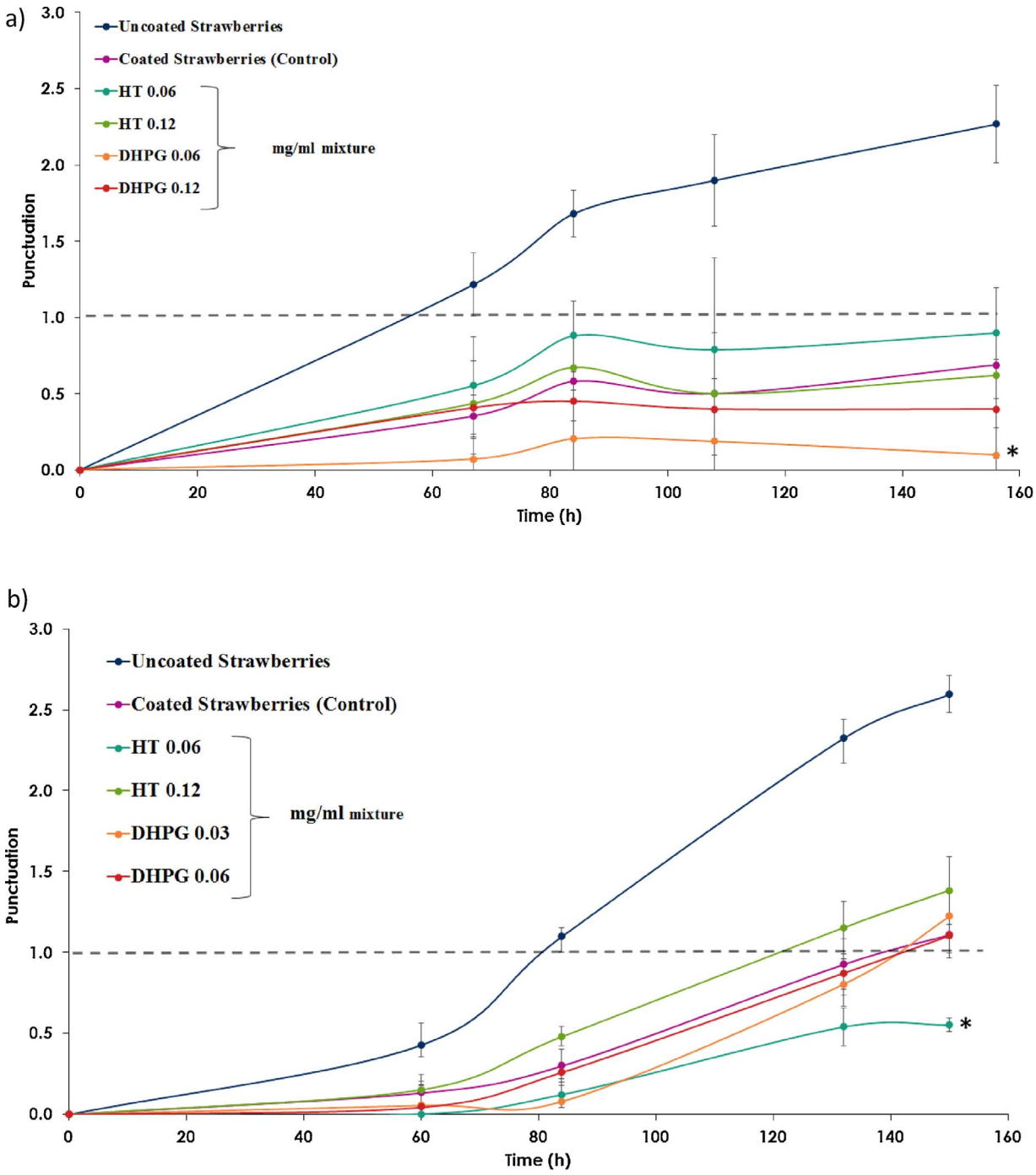

Fig. 5. Average punctuation of decay for strawberries covered with an edible pectin-fish skin protein film formulation containing hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) individually at two different experiments carried out at different months (a) and (b). Comparison with uncoated strawberries and with coated strawberries without bioactive compounds (control). The concentration of plasticizer utilized was 0.5 g of glycerol/g polymer. * Significant difference at 157 h compared to the uncoated strawberries and the control ($p < 0.05$).

bioactive films with double concentration of plasticizer.

These results demonstrate the potential use of the natural phenolic compounds from olive, HT and DHPG, as antifungal coatings against a wide range of storage fungi that affect strawberry perishability. The strawberries coated with pectin-protein-composite film also presented a significantly lower amount of mold growth than uncoated strawberries independently of the concentration of plasticizer used, although the film with a higher concentration of plasticizer was more effective at delaying the decay of stored strawberries. Importantly for consumers, the phenolic compounds did not significantly affect the surface color of the strawberries (Table 2).

5. Conclusions

The film containing bioactive compounds, HT and DHPG, had a marked mechanical resistance, showing the highest stretching capacity and resistance to breakage. Also, the WVP and oxygen permeability of the pectin-protein film was not affected by the incorporation of HT-DHPG, and the bioactive films exhibited good barrier properties. The addition of more glycerol improved film stiffness with a fall in the elastic modulus but compromised the film's barrier properties.

The release of HT and DHPG from the film improved the presence of natural antioxidant in strawberries or other foodstuffs. The bioactive

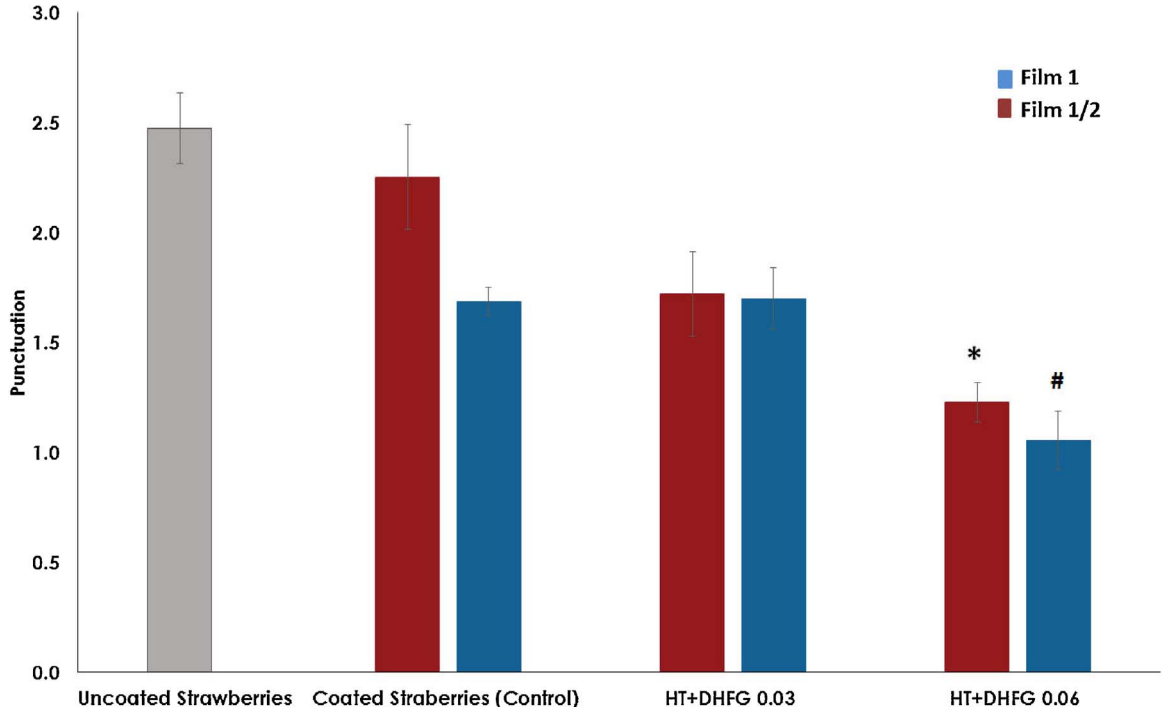

Fig. 6. Average punctuation of decay for strawberries covered with an edible pectin-fish skin protein film-forming formulation with two concentrations of glycerol added (Film 1 and Film 1/2) and containing a mixture of HT: DHPG (1:1) at 0.03 and 0.06 mg/ mL during storage at room temperature for six days. Error bars indicate standard deviations. * Significant differences compared to uncoated strawberries, the control and the other concentration ($p < 0.05$). # Significant difference between Film 1 and Film 1/2 at concentration of HT 0.06 + DHPG 0.06 ($p < 0.05$).

edible coating was effective at delaying mold growth on strawberries during storage and to extend the shelf-life of fresh strawberries. Also, the bioactive films may have potential health benefits upon consumption. Alternatively, the films could act as a potential vehicle for the delivery of the bioactive compounds HT and DHPG, with potential applications in the biomedical field.

Acknowledgements

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References

ASTM (American Society for Testing and Materials) (2002). *D882. Standard test method for tensile properties of thin plastic sheeting.* ASTM.

ASTM (American Society for Testing and Materials) (2014). *F1927. Standard test method for determination of oxygen gas transmission rate, permeability and permeance at controlled relative humidity through barrier materials using a coulometric detector.* ASTM.

ASTM (American Society for Testing and Materials) (2016). *E-96 standard test methods for water vapor transmission of materials.* ASTM.

Arrieta, M. P., Peltzer, M. A., Garrigós, M. C., & Jiménez, A. (2013). Structure and mechanical properties of sodium and calcium caseinate edible active film with carvacrol. *Journal of Food Engineering, 114*, 486–494.

Barkay-Olami, H., & Zilberman, M. (2016). Novel porous soy protein-based blend structures for biomedical applications: Microstructure, mechanical, and physical properties. *Journal of Biomedical Materials Research Part b-applied Biomaterials, 104*, 1109–1120.

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Lama-Muñoz, A., & Fernández-Bolaños, J. (2017). Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting. *Carbohydrate Polymers, 163*, 292–300.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*, 484–489.

Bradford, M. M. (1976). A Rapid and sensitive method for the quantification of microgram cuantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Cuq, B., Gontard, N., Cuq, J. L., & Guilbert, S. (1997). Selected functional properties of fish myofibrillar protein-based films as affected by hydrophilic plasticizers. *Journal of Agricultural and Food Chemistry, 45*, 622–626.

De Roos, B., Zhang, X., Rodríguez Gutiérrez, G., Wood, S., Rucklidge, G. J., Reid, M. D., et al. (2011). Anti-platelet effects of olive oil extract: In vitro functional and proteomic studies. *European Journal of Nutrition, 50*, 553–562.

Di Pierro, P., Mariniello, L., Giosafatto, C. V. L., Masi, P., & Porta, R. (2005). Solubility and permeability properties of edible pectin-soy flour films obtained in the absence or presence of transglutaminase. *Food Biotechnology, 19*, 37–49.

Di Pierro, P., Marquez, G. R., Mariniello, L., Sorrentino, A., Villalonga, R., & Porta, R. (2013). Effect of transglutaminase on the mechanical and barrier properties of whey protein/pectin films prepared at complexation pH. *Journal of Agricultural and Food Chemistry, 61*, 4593–4598.

Eça, K. S., Machado, M. T. C., Hubinger, M. D., & Menegalli, F. C. (2015). Development of active films from pectin and fruit extracts: Light protection, antioxidant capacity, and compounds stability. *Journal of Food Science, 80*, 2389–2396.

Farris, S., Schaich, K. M., Liu, L., Cooke, P. H., Piergiovanni, L., & Yam, K. L. (2009). Development of polyion-complex hydrogels as an alternative approach for the production of bio-based polymers for food packaging: A review. *Trends in Food Science & Technology, 20*, 316–332.

Farris, S., Schaich, K. M., Liu, L., Cooke, P. H., Piergiovanni, L., & Yam, K. L. (2011). Gelatin-pectin composite films from polyion-complex hydrogels. *Food Hydrocolloids, 25*, 61–70.

Fernández-Bolaños, J., Guillén, R., Jiménez, A., Rodríguez, R., Rodríguez-Gutiérrez, G., & Lama-Muñoz, A. (2011). *Method for purifying 3,4-Dihydroxyphenylglycol (DHPG) from plant products*. International publication number WO 2010/070168.

Fernández-Bolaños, G. J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Rubio-Senent, F., Fernández-Bolaños, G. J. M., Maya, I., et al. (2014). *Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroyphenylglycol extract, and hydroxytyrosol acetate extract, from by-products of the olive tree, and the purification thereof*. International publication number WO 2013/007850.

Hu, T., He, X. W., Jiang, J. G., & Xu, X. L. (2014). Hydroxytyrosol and its potential therapeutic effects. *Journal of Agricultural and Food Chemistry, 62*, 1449–1455.

Khalifa, I., Barakat, H., E-Mansy, A. H., & Soliman, A. S. (2016). Improving the shelf-life stability of apple and strawberry fruits applying chitosan-incorporated olive oil processing residues coating. *Food Packaging and Shelf Life, 9*, 10–19.

Killeen, M. J., Pontoniere, P., & Crea, R. (2011). Hydroxytyrosol: An examination of its potential role in cardiovascular disease, inflammation, and longevity. *AgroFood Industry Hi-Tech, 22*, 16–19.

Liu, L. S., Fishman, M. L., & Hicks, K. B. (2007). Composite films from pectin and fish skin gelatin or soybean flour protein. *Journal of Agricultural and Food Chemistry, 55*, 2349–2355.

Liu, L. S., Kost, J., Yan, F., & Spiro, R. C. (2012). Hydrogels from biopolymer hybrid for biomedical, food, and functional food applications. *Polymers, 4*, 997–1011.

Ng, S. F., & Tan, S. L. (2015). Development and in vitro assessment of alginate bilayer

films containing the olive compound hydroxytyrosol as an alternative for topical chemotherapy. *International Journal of Pharmaceutics, 495*, 798–806.
Ng, S. F., Tan, L. S., & Buang, F. (2017). Transdermal anti-inflammatory activity of bilayer film containing olive compound hydroxytyrosol: Physical assessment, in vivo dermal safety and efficacy study in Freund's adjuvant-induced arthritic rat model. *Drug Development and Industrial Pharmacy, 43*, 108–119.
Nuñez-Flores, R., Giménez, B., Fernández-Martín, M. E., López-Caballero, M. E., Montero, M. P., & Gómez-Guillén, M. C. (2013). Physical and functional characterization of active fish gelatin films incorporated with lignin. *Food Hydrocolloids, 30*, 163–172.
Porta, R., Di Pierro, P., Sabbah, M., Regalado-Gonzales, C., Mariniello, L., Kadivar, M., et al. (2016). Blend films of pectin and bitter vetch (*Vicia ervilia*) proteins: Properties and effects of transglutaminase. *Innovative Food Science and Emerging Technologies, 36*, 245–251.
Prodpran, T., Benjakul, S., & Phatcharat, S. (2012). Effect of phenolic compounds on protein cross-linking and properties of film from fish myofibrillar protein. *International Journal of Biological Macromolecules, 51*, 774–782.
Rodríguez-Gutiérrez, G., Duthie, G. G., Wood, S., Morrice, P., Nicol, F., Reid, M., et al. (2012). Alperujo extract, hydroxytyrosol, and 3, 4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats – a proteomics and network analysis approach. *Molecular Nutrition and Food Research, 56*, 1137–1147.
Rojas-Graü, M. A., Avena-Bustillos, R. J., Friedman, M., Henika, P. R., Martín-Bellosos, O., & McHugh, T. H. (2006). Mechanical, barrier, and antimicrobial properties of Apple puree edible films containing plant essential oils. *Journal of Agricultural and Food Chemistry, 54*, 9262–9267.
Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from alperujo and their antioxidant activities. *Journal Agricultural and Food Chemistry, 60*, 1175–1186.
Yangui, T., Dhouib, A., Rhouma, A., & Sayadi, S. (2009). Potential of hydroxytyrosol-rich composition from olive mill wastewater as a natural disinfectant and its effect on seeds vigour response. *Food Chemistry, 1*, 1–8.
Yangui, T., Sayadi, S., Rhouma, A., & Dhouib, A. (2010). Potential use of hydroxytyrosol-rich extract from olive mill wastewater as a biological fungicide against Botrytis cinerea in tomato. *Journal of Pest Science, 83*, 437–445.

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Effect of edible pectin-fish gelatin films containing the olive antioxidants hydroxytyrosol and 3,4-dihydroxyphenylglycol on beef meat during refrigerated storage

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ABSTRACT

The objective of this research was to evaluate the effect of the addition of two antioxidants naturally present in olives, hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), to a pectin-fish gelatin edible film on the preservation of raw beef meat during refrigerated storage. A new composite film that included beeswax was also prepared, resulting in a reduction in the film's oxygen permeability. Results showed that the meat samples wrapped with film containing antioxidants reduced the formation of oxidation products in the form of thiobarbituric acid reaction substances (TBARS) compared with control film without antioxidants. HT added at 0.5% to the film with beeswax suppressed the lipid oxidation of beef meat during 7 days of storage at 4 °C, possibly by the combined effect of acting as an oxygen barrier and the specific antioxidant activity. The interference of plasticizer agents (glycerol and sorbitol) incorporated to the film on the TBARS method was showed for the first time.

1. Introduction

Lipid oxidation is the major contributing factor to the deterioration of raw meat during storage, causing a reduction of quality and acceptability due to the development of an undesirable flavor and rancidity (Nielsen, Sørensen, Skibsted, & Bertelsen, 1997). Lipid oxidation can be controlled by the use of antioxidants. However, due to the potential health hazard of some synthetic antioxidants commonly used in the food industry, like butyl hydroxyanisole (BHA) or butyl hydroxytoluene (BHT), it may be desirable to replace these conventional antioxidants with natural anti-oxidative products.

Olives (*Olea europeae*) and olive oil contain numerous polyphenols with excellent antioxidant properties. However, most phenols (about 98%) remain in the liquid-solid waste called *alperujo* that remains after the extraction of olive oil and only 2% are transferred to the oil. Therefore, alperujo is an abundant source of natural antioxidants. This olive oil by-product offers a practical and economic source of antioxidants that could replace synthetic substances for use in the food industry (Fernández-Bolaños, López, Fernández-Bolaños, & Rodríguez-Gutiérrrez, 2008). In particular, hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) are two individual phenols abundant in olive fruit and olive oil with important antioxidant properties and their positive effects have been demonstrated on human health (Ciriminna, Meneguzzo, Fidalgo, Ilharco, & Pagliaro, 2016; Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007).

Olive extract and hydroxytyrosol have been widely used in the food industry and many authors have reported their effectiveness in the reduction of the lipid oxidation of meat (Cofrades et al., 2011; DeJong & Lanari, 2009) and fish (Medina, Sacchi, Biondi, Aubourg, & Paolillo, 1998; Medina, Satué-Gracia, Bruce German, & Frankel, 1999; Pazos, Alonso, Sánchez, & Medina, 2008). In contrast, few studies have been conducted until now on the functional properties of DHPG, an ortho-diphenol structurally similar to HT, with an additional hydroxyl in the β position. Moreover, most studies focused on the lipid oxidation of meat or fish involve the direct addition of HT or phenolic extract to the muscle. To the best of our knowledge, the use of HT and DHPG as antioxidant components of edible films has not previously been reported.

Edible films and coatings help maintain the quality of meat products during their storage, mainly as a result of their ability to act as a water barrier, preventing dehydration, and an oxygen barrier, reducing lipid oxidation (Bonilla, Atarés, Vargas, & Chiralt, 2012; Gennadios, Hanna,

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& Kurth, 1997). Furthermore, antioxidants can be added to the film formulation leading to better preservation since a high concentration of antioxidant is present on the surface of the meat (Gomez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009).

Films made from proteins and carbohydrates are excellent barriers to oxygen because of their tightly packed, ordered hydrogen-bonded network structure. However, in general, due to their hydrophilic nature, polymeric films generally exhibit limited water vapor permeability (WVP) barrier ability.

Pectin is a water-soluble hygroscopic polymer that is commonly used in the food and pharmaceutical industries as a thickening, coating and encapsulating material due to its gelling, stabilizing and thickening properties (Ahn, Halake, & Lee, 2017). However, relatively few studies have reported on the use of pectin as a source of edible coating for meat patties (Kang et al., 2007).

Gelatin is a protein based polymer widely used as a starting material for edible film formation. However, fish gelatin film showed poor water resistance properties (Nilsuwan, Benjakul, & Prodpran, 2016). Pectin-gelatin blended film offers advantages in terms of its mechanical properties and WVP with respect to films formed from pectin or protein alone (Di Pierro et al., 2013). In a previous work, we developed a pectin-gelatin film enriched with olive phenolic compounds HT or DHPG, with good mechanical and functional properties that extended the shelf life of strawberries against molds with a significant delay in visible decay (Bermúdez-Oria, Rodríguez-Gutiérrez, Vioque, Rubio-Senent, & Fernández-Bolaños, 2017). This biopolymer-based film, combined with HT or DHPG, was an excellent oxygen barrier; however, its WVP was limited due to its hydrophilic nature. In this work, beeswax, a hydrophobic substance, was incorporated to assess the efficiency of the composite film to water-vapor transmission. Also, in the present study the development of pectin plus protein composite films with antioxidant capacity was studied in relation to meat preservation. The aim of this study was to evaluate and compare the antioxidant effect of two olive phenolic compounds (HT and DHPG), when added to pectin-fish gelatin films, on the oxidative stability of beef during refrigerated storage.

2. Materials and methods

2.1. Materials

HT and DHPG were extracted and purified from a by-product obtained from manufacturing of olive oil by a chromatographic system based on ion exchange, as described by Fernández-Bolaños et al. (2011) and Fernández-Bolaños et al. (2014). Citrus pectin with a high degree of esterification (53%), gelatin from fish skin (~95%), beeswax, and sorbitol were purchased from Sigma-Aldrich (St Louis, MO, USA). Glycerol was purchased from Panreac Química, S.A. (Barcelona, Spain). Commercial film of low-density polyethylene was acquired in a local supermarket.

2.2. Film preparation

Films were prepared by dissolving 0.5 g of citrus pectin and 0.5 g of fish gelatin in 20 mL of distilled water with continuous stirring at room temperature for 2 h. The pH of the mixture was first adjusted to 8 using 4 M NaOH then gradually reduced to 3–4 with 1 M HCl. To the polymer solution, 0.5 g/g glycerol was added as a plasticizer and the mixture was stirred vigorously for 30 min. For the film with beeswax, 0.4 g/g beeswax was heated to 70 °C until it melted and added to the polymer. The mixture was heated to 70 °C and stirred vigorously for 15 min to allow correct homogenization of the beeswax mixture. After complete dispersion, the phenolic compounds HT and DHPG were added to the mixture at a concentration of 0.1–0.5% HT and DHPG with respect to dry polymers. Air bubles were removed from the resultant gel by vacuum in kitasato during 1 h. The resultant gel was degassed and spread gently and very slowly on the surface of polypropylene film to avoid the formation of bubbles and then left to dry for 48 h at room temperature. The same methodology was used to prepare films containing no phenolic compounds (film control, without the addition of HT and DHPG).

2.3. Film thickness

Film thickness, expressed in μm, was measured using a micrometer (Baxlo 4000/Film) with an accuracy of 0.001 mm. Measurements were taken at five random sites on each film and for five films of each formulation. The mean and standard deviation were calculated for the evaluation of mechanical properties.

2.4. Oxygen permeability

The oxygen permeability (OP) of the films was analyzed using an oxygen transmission rate system (Mocom, USA) following the standard American Society for Testing and Materials method (ASTM, 2014). An effective area of 50 cm^2 was exposed to permeation in test conditions of 25 °C and 50% relative humidity. Assays were performed in triplicate and the mean values were expressed as $cm^3 \cdot \mu m/m^2 \cdot d \cdot KPa$.

2.5. Water vapor permeability

Water vapor permeability (WVP) was determined following the gravimetric method (ASTM, 2016). Film samples were sealed over a circular opening of an aluminum permeation container filled with anhydrous $CaCl_2$. The cells were kept in a hermetically closed chamber containing an oversaturated solution of Mg $(NO_3)_2$ at 22 to 25 °C in order to maintain a relative humidity difference of 95%. The WVP was determined from the slope obtained for the regression analysis of weight loss data versus time, once the steady state had been reached, divided by the film area. Results were expressed as $g \cdot mm/h \cdot m^2 \cdot kPa$. All tests were carried out at least in triplicate. An empty aluminum cup covered with the film was used as control.

2.6. Solubility of film components and bioactive compounds

All films were pre-dried in a desiccator for 48 h prior to testing. The solubility of the composite films was tested in water at different pH values. Specimens (4 × 2 cm) were placed in 50 mL of 0.1 M acetate (pH 4.0), phosphate (pH 7.2), or Tris-HCl (pH 8.5) buffer solutions for 1, 24 and 48 h. The rest of the specimens were removed, and the amounts of pectin and protein from the films that had solubilized into the different solutions were measured by the uronic acid assay (Blumenkrantz & Asboe-Hansen, 1973) and Bradford assay (Bradford, 1976), respectively. The HT/DHPG released in distilled water was determined by HPLC according to a previously published method (Rodríguez et al., 2007).

2.7. Preparation of meat samples

Fresh beef meat was purchased from a local market. The beef samples were obtained from topside muscle containing about 4.3 g of fat/100 g muscle. The beef meat was divided in pieces of 7 g (2 × 2 × 3 cm) and packaged with commercial film, with film without antioxidant or film with different concentrations of HT and DHPG and stored at 4 °C for 6–7 days. One sample was packed for each trial day.

2.8. Thiobarbituric acid reactive substances (TBARS) assay

Lipid oxidation was measured by the 2-thiobarbituric acid extraction method of Witte, Krause, and Bailey (1970) with modifications. Briefly, 1 g of meat sample was mixed with 3 mL of trichloroacetic acid (TCA) (75 g/L) and 10 μL of t-butyl-4-hydroxyanisole and homogenized using a homogenizer (Ultra-Turrax®T50 Basic, IKA, Germany) at a

speed of 24,000 rpm for 2 min in an ice bath. The resultant slurry was centrifuged at 5900*g* for 5 min and filtered through a 0.45 μm membrane. To a glass tube containing 1 mL of thiobarbituric acid (2.88 g/L), 1 mL of filtrate was added. The tubes were closed and heated at 80 °C in a water bath for 90 min. TBARS values were calculated using the absorbance of each sample at 532 nm and a standard curve was constructed using 1,1,3,3-tetraethoxypropane (1–5 mg/L). TBARS values were expressed as mg malonaldehyde/kg meat (MDA mg/kg meat). All measures were carried out in triplicate.

2.9. Statistical analysis

Results were expressed as mean values ± standard deviations. STATGRAPHICS® plus software was used for statistical analysis. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) and the LSD method. A *p*-value of 0.05 was considered significant.

3. Results and discussion

3.1. Formation of thiobarbituric acid reactive substances (TBARS)

The pectin-gelatin based films, with and without beeswax added, containing natural antioxidant (HT or DHPG) were evaluated for their effectiveness as a preservative coating against oxidative changes to beef meat during a storage period of 6–7 days at 4 °C. Lipid oxidation of raw beef meat was monitored by measuring the formation of thiobarbituric acid and reactive substances (TBARS), the secondary products of lipid oxidation, such as malonaldehyde (MDA), which forms a pink color upon reaction with thiobarbituric acid.

The presence of a significant amount of hydrophilic plasticizer, such as glycerol, incorporated into the film to impart adequate flexibility, showed a significant interference with thiobarbituric acid, whose reaction produced a red color at the concentration (0.5 g/g of polymer) utilized in our initial film preparation studies (Bermúdez-Oria et al., 2017). We also assayed the formation of film with other plasticizer agents such as sorbitol, which showed even greater interference than glycerol (Fig. 1a). To the best of our knowledge, this is the first time that plasticizer interference with thiobarbituric acid has been reported.

Since the film was very hygroscopic, it was not easy to remove the film completely from the meat, a high moisture product, to make the measurement and we found that the glycerol/sorbitol could migrate into the food with ease. As a result of this interference, we observed an increase in TBARS values, which would mask the antioxidant effect of the compounds and could explain certain apparently inconsistent results from other studies (Coşkun, Çalikoğlu, Emiroğlu, & Candoğan, 2014; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004). Thus, the corresponding absorbance of the plasticizer was subtracted in all the determinations to avoid this interference. Two portions of the pectin-gelatin film (1/8 and 1/16) with and without beeswax incorporated (Fig. 1b), corresponding with two concentrations of glycerol and sorbitol (mg/mL of TCA) as plasticizer, were assayed using the TBARS method to assign a reference value to be subtracted. The data revealed that a higher concentration of plasticizer in the film formulation produced a greater difference in absorbance: sorbitol + beeswax < sorbitol ~ glycerol + beeswax > glycerol. However, at low plasticizer concentration, there were practically no differences between glycerol and sorbitol or the addition of beeswax.

Next, we evaluated the influence of the antioxidant doses 0.1 and 0.5 g HT or DHPG/100 g of polymer, corresponding to 100 or 500 mg of antioxidant/kg of meat, on the TBARS values of raw beef. Antioxidant incorporation into the edible films was effective against lipid oxidation (Fig. 2). The pectin-gelatin control film without antioxidant revealed that the TBARS values increased significantly ($p < 0.05$) during the meat's storage. A similar result was observed for the commercial polypropylene film, with a somewhat smaller increase. TBARS values of

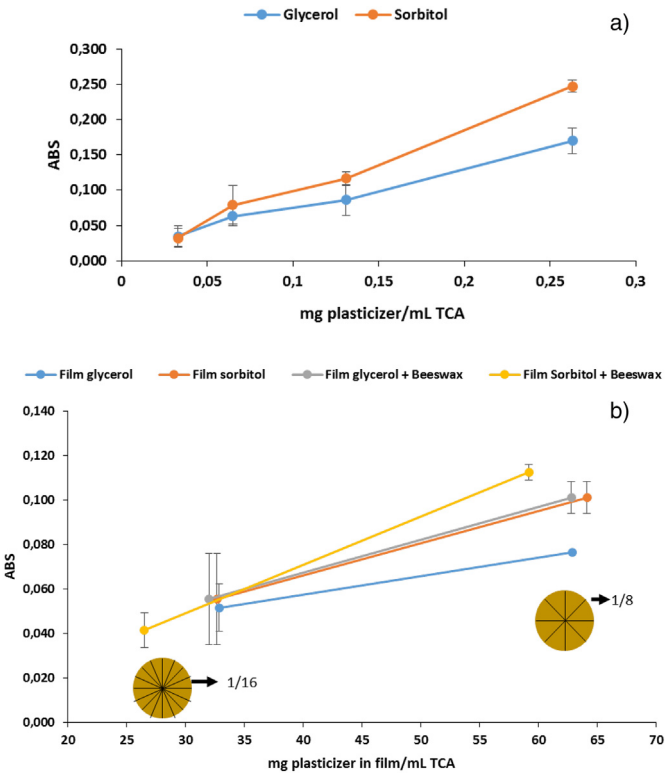

Fig. 1. Effect of plasticizer (mg/mL) on the absorbance value of TBARS method. (a) Influence of plasticizer incorporated in the edible pectin-gelatin film, with and without beeswax added, on the TBARS absorbance value. (b) Portions of 1/16 and 1/8 of a film with 3 g of polymer containing 1.5 g of glycerol or sorbitol were solubilized in 3 mL of trichloroacetic acid.

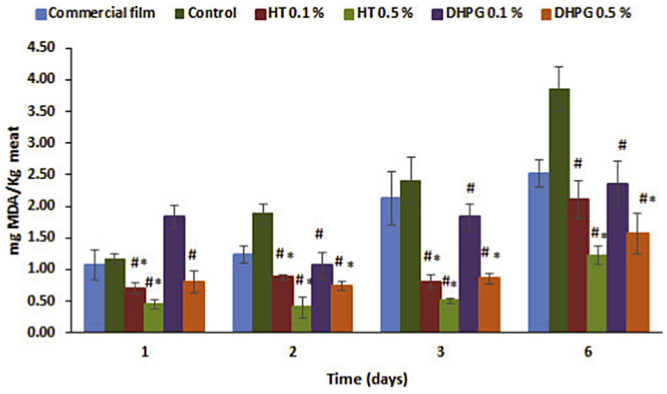

Fig. 2. Thiobarbituric acid reaction substances (TBARS) (mg malonaldehyde (MDA)/kg meat) in beef samples coated with edible pectin-gelatin film containing two concentrations of hydroxytyrosol (HT) or 3,4-dihydroxyphenylglycol (DHPG), compared to meat protected by film without antioxidants (control) and meat protected with a commercial polyethylene film, during storage at 4 °C for 1 to 6 days. All studies were carried out in duplicate. Means for each storage period marked with * and # are statistically different ($p < 0.05$) with respect to commercial film and control film, respectively.

the antioxidant film-covered beef were stable during the first three days of storage followed by a significant increase ($p < 0.05$) of lipid oxidation at day 6 at both doses assayed. Nevertheless, there was an improved lipid stabilization of 68% for HT (0.5%) and of 59% for DHPG (0.5%) compared to the control sample film without antioxidant. Moreover, the effectiveness of HT on TBARS in beef meat was more important than DHPG. This result is in contrast to a previous study, which showed that DHPG was a more effective antioxidant than HT (Rodríguez-Gutiérrez et al., 2012). A film containing a mixture of HT

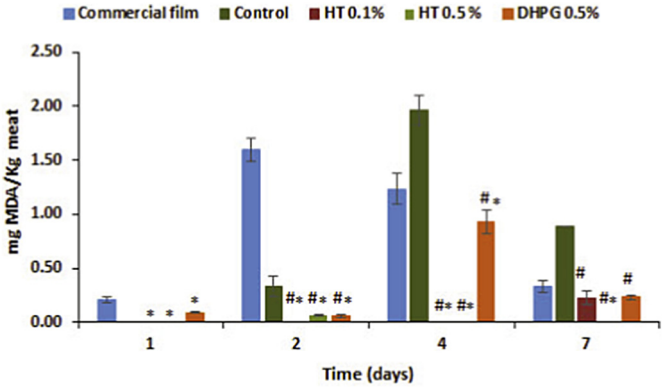

Fig. 3. Thiobarbituric acid reaction substances (TBARS) (mg MDA/kg meat) in beef samples with edible pectin-gelatin film containing beeswax and hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) as active compounds, a control of meat with film without antioxidants, and a meat with a film commercial of polyethylene, during storage at 4 °C for 7 days. All studies were carried out in duplicate. Means for each storage period marked with *, # are statistically different ($p < 0.05$) with respect to commercial film and control film, respectively.

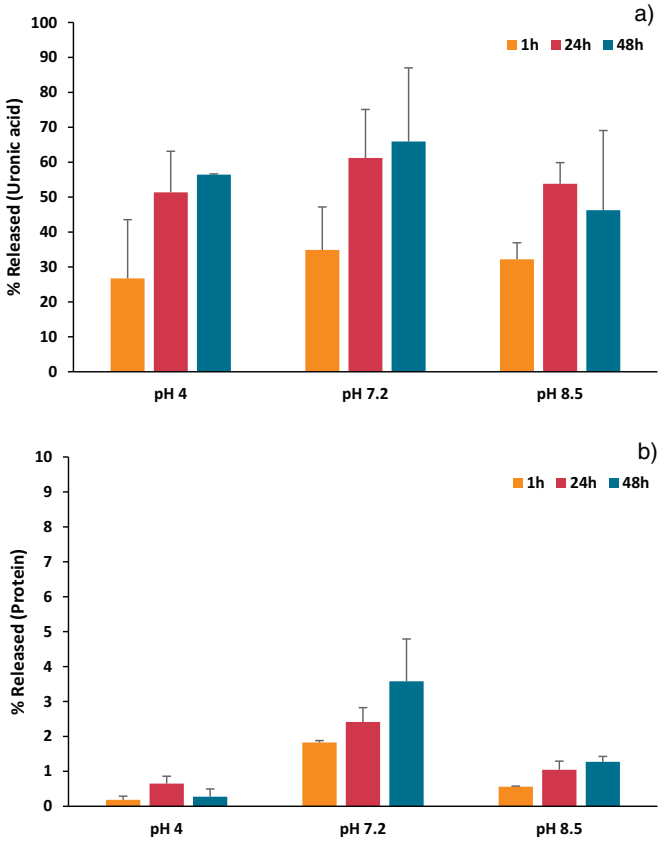

Fig. 4. Release (%) of pectin (a) and protein (b) from pectin-skin fish protein composite film with beeswax added at different pHs (4, 7.2, and 8.5) after 1, 24, and 48 h incubation at room temperature. Results are expressed as mean values and the bars indicate standard deviation.

and DHPG did not improve the reduction of TBARS compared to film with the individual antioxidants (data not shown). These results showed that the incorporation of natural antioxidants HT and DHPG in the pectin-gelatin film improved the protection of meat samples against lipid oxidation. The direct application of these compounds alone, as dip solution at the same concentration that in film, reduced the lipid oxidation only until 3 days.

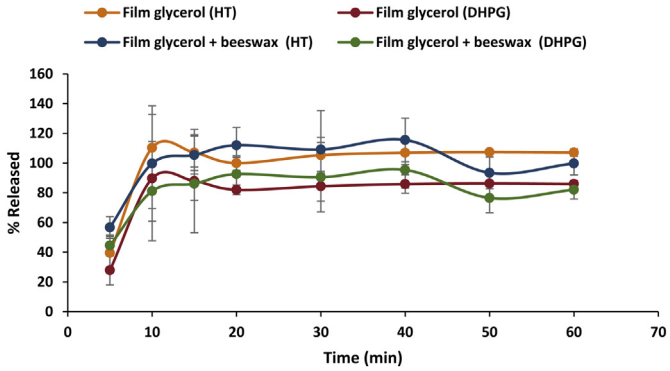

Fig. 5. Release (%) of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from pectin-skin fish protein composite film with and without beeswax in distilled water for 60 min. Results are expressed as mean values and the bars indicate standard deviation.

The protective effect of antioxidants incorporated into a second film of pectin-gelatin with beeswax added for improved moisture transfer retardation was evaluated by the determination of TBARS (Fig. 3). The meat wrapped with commercial film of low density polyethylene without antioxidant added showed an increase in TBARS formation between 24 and 48 h storage, reaching a maximum of 1.6 mg malonaldehyde/kg of meat. A significant reduction in values was then observed from day 4 to the end of the 7-day storage period. A similar evolution was observed for the control pectin-gelatin based film sample without antioxidants, reaching a maximum of 1.9 mg malonaldehyde/kg of meat at day 4 of storage. HT and DHPG incorporation significantly ($p < 0.05$) reduced the formation of TBARS compared with the control film. No significant change in the TBARS levels of HT-coated beef meat were detected during the whole storage period; 0.5 g of HT/100 g of polymer film totally suppressed lipid oxidation even after 7 days of storage. The only significant difference ($p < 0.05$) was observed between HT and DHPG on day 4 of storage. This fact confirmed the result of the first experiment that HT is a more effective antioxidant than DHPG for protecting beef meat from lipid oxidation.

The results of this study show that all the active films tested exhibited a protective effect on the lipid oxidation of beef. Also, HT showed an even greater antioxidant capacity when incorporated in film containing beeswax.

3.2. Film characterization

Our initial study (Bermúdez-Oria et al., 2017) on a composite edible film based on pectin and fish skin protein, with glycerol added as a plasticizer and HT or DHPG as active agents, showed that the film had adequate physical properties including a marked mechanical resistance and a good barrier to oxygen permeability (11.2 cm^3 µm/m^2 d kPa). However, the composite film did not reduce moisture loss and thus it was necessary to incorporate hydrophobic substances such as beeswax to assess the efficacy of the film as a barrier to water. However, the results obtained still showed a poor permeability for water, and no significant differences were found between the film with or without beeswax. Nevertheless, the addition of beeswax improved the oxygen permeability, reducing this to a value of 8.92 ± 1.11 cm^3 µm/m^2 d kPa, a significant difference compared to the film without beeswax.

In this new composite film prepared with beeswax and HT or DHPG, we studied the release of film components (pectin and protein) and the bioactive compounds. The release of pectin and protein was measured after 1, 24 and 48 h of incubation at different pHs (4, 7.2 and 8.5) (Fig. 4). There were no significant differences between the release of pectin at the different pHs. For the film with beeswax, a slight decrease was observed in the release of solubilized pectin with respect to the film without beeswax (Bermúdez-Oria et al., 2017). Film without beeswax

Table 1
Antiradical activity (DPPH·) of the film containing a mixture of HT and DHPG during storage in a desiccator during 8 weeks.

	mmolTrolox/ g of theoretical mixture HT + DHPG in film			
	Week 0	Week 1	Week 3	Week 8
Film (pectin-fish gelatin + glycerol)	6.25 ± 0.026[a]	8.71 ± 0.143[b]	9.64 ± 0.791[b]	12.9 ± 0.589[c]
Film (pectin-fish gelatin + glycerol + beeswax)	5.62 ± 0.525[a]	7.48 ± 0.892[a]	8.51 ± 1.691[a]	13.7 ± 0.861[b]

Values are expressed as millimoles of Trolox per g of theoretical mixture of HT + DHPG present in the film. The radical scavenging capacity of HT + DHPG by the DPPH· method was 6.13 ± 0.10 mmolTrolox /g of HT + DHPG. Values are the means of a duplicate assay. Means bearing the same letter are not significantly different at the 5% level.

had a maximum release of pectin after 24 h incubation. While the film without beeswax had lower, pectin solubility at pH 4 and almost 100% of uronic acid was released at pH 7.2 following 48 h incubation. The amount of solubilized protein decreased significantly as pectin was released The solubility of protein at pH 4 was practically null, which is in agreement with the formation of an electrostatic complex at a pH close to pectin's pKa (3.55–4.10) and protein's pI (4.8–5.2) (Liu, Fishman, & Hicks, 2007).

The release of phenolic compounds, HT and DHPG, from the two films with and without beeswax was also evaluated after a 60 min incubation in distilled water (Fig. 5). The two film formulations showed a peak of phenol solubilization by 10 mins, with the complete release of HT at 10 min and 80% release of DHPG, with % release values for both remaining constant until 60 min. Therefore, the active migration of antioxidant compounds from the film to the meat, a high moisture product, should be quick and easy. However, it was not easy to remove the film completely from the meat to determine the amount of active compound into the product, although it can be assumed that the active compounds pass to the meat in approximately 10 min.

The availability and stability of the bioactive compounds in the films were evaluated by the HPLC determination of HT and DHPG concentration present in the film during storage for up to 8 weeks in a desiccator. For this experiment, a film containing a mixture of HT and DHPG with and without beeswax was tested. The amounts of HT and DHPG remained constant in both films during 8 weeks of storage (results not shown). These results were consistent in part with those obtained with the DPPH· method (Table 1). However, curiously, the antiradical activity increased ($p < 0.05$) with the time of storage, from 5.62 and 6.25 mmol Trolox/g of antioxidant mixture in the film at week 0 to 13.7 and 12.9 mmol Trolox/g antioxidant at week 8 for films with and without beeswax, respectively (Table 1). Maillard reaction between gelatin and pectin, or its degradation products (Wegener, Bornik, & Kroh, 2015), or between gelatin and glycerol (Zhou, Guo, Liu, Liu, & Labuz, 2013) during the storage stage at room temperature could explain this increase of antiradical activity by the formation of antioxidant activity of Maillard reaction products (Phisut & Jiraporn, 2013).

4. Conclusions

The bioactive edible film made of pectin and fish gelatin containing the natural antioxidants, HT or DHPG, was effective at delaying the lipid oxidation of raw beef meat during refrigerated storage. The use of these compounds, with several beneficial effect in health (anti-inflammatory, anticancer, protector cardiovascular, prevention of diabetes, etc) is a good strategy to replace synthetic preservatives in meat. Also, in previous studies, this film exhibited adequate mechanical and oxygen barrier properties (Bermúdez-Oria et al., 2017). This study demonstrated that the addition of beeswax to the film formulation improved the oxygen barrier property and enhanced the oxidative stability of beef relative to the film control, without natural antioxidant, during storage at 4 °C. The best protective effect was obtained for film containing HT and beeswax, which reduced lipid oxidation by 100% during 7 days, possibly by the combined effect of the film acting as an oxygen barrier and the antioxidant protection of HT. An edible active film with these antioxidants might have a double positive benefit; one on the oxidation stability of the meat fat itself, and a second health benefit for the consumer. However, very in-depth studies would be required to confirm this secondary benefit as these preliminary results are promising, additional research will be required to improve the formulation to optimize the film's moisture content as well as the sensory properties of meat treated with edible film containing phenolic compounds present in olive fruit. Therefore, to get an edible active film with these antioxidants had a double benefits, a positive effect on the oxidation stability of meat fat and beneficial effect in the human organism.

Declaration of interest

None.

Acknowledgements

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References

Ahn, S., Halake, K., & Lee, J. (2017). Antioxidant and ion-induced gelation functions of pectins enabled by polyphenol conjugation. *International Journal of Biological Macromolecules, 101*, 776–782.
ASTM (American Society for Testing and Materials) (2014). *F1927. Standard test method for determination of oxygen gas transmission rate, permeability and permeance at controlled relative humidity through barrier materials using a coulometric detector*.
ASTM (American Society for Testing and Materials) (2016). *E-96. Standard test methods for water vapor transmission of materials*.
Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Vioque, B., Rubio-Senent, F., & Fernández-Bolaños, J. (2017). Physical and functional properties of pectin-fish gelatin films containing the olive phenols hydroxytyrosol and 3,4-dihydroxyphenylglycol. *Carbohydrate Polymers, 178*, 368–377.
Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*(2), 484–489.
Bonilla, J., Atarés, L., Vargas, M., & Chiralt, A. (2012). Edible films and coatings to prevent the detrimental effect of oxygen on food quality: Possibilities and limitations. *Journal of Food Engineering, 110*(2), 208–213.
Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.
Ciriminna, R., Meneguzzo, F., Fidalgo, A., Ilharco, L. M., & Pagliaro, M. (2016). Extraction, benefits and valorization of olive polyphenols. *European Journal of Lipid Science and Technology, 118*(4), 503–511.
Cofrades, S., Salcedo Sandoval, L., Delgado-Pando, G., López-López, I., Ruiz-Capillas, C., & Jiménez-Colmenero, F. (2011). Antioxidant activity of hydroxytyrosol in frankfurters enriched with n-3 polyunsaturated fatty acids. *Food Chemistry, 129*(2), 429–436.
Coşkun, B. K., Çalikoğlu, E., Emiroğlu, Z. K., & Candoğan, K. (2014). Antioxidant active packaging with soy edible films and oregano or thyme essential oils for oxidative stability of ground beef patties. *Journal of Food Quality, 37*(3), 203–212.
DeJong, S., & Lanari, M. C. (2009). Extracts of olive polyphenols improve lipid stability in cooked beef and pork: Contribution of individual phenolics to the antioxidant activity of the extract. *Food Chemistry, 116*(4), 892–897.
Di Pierro, P., Rossi Marquez, G., Mariniello, L., Sorrentino, A., Villalonga, R., & Porta, R. (2013). Effect of transglutaminase on the mechanical and barrier properties of whey

protein/pectin films prepared at complexation pH. *Journal of Agricultural and Food Chemistry, 61*(19), 4593–4598.
Fernández-Bolaños, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Rubio-Senent, F., Fernández-Bolaños Guzmán, J. M., Maya, I., ... Marset, A. (2014). *Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroyphenylglycol extract, and hydroxytyrosol acetate extract, from by-products of the olive tree, and the purification thereof.* (International Patent publicationnumber WO/2013/007850).
Fernández-Bolaños, J., Guillén, R., Jiménez, A., Rodríguez, R., Rodríguez-Gutiérrez, G., & Lama-Muñoz, A. (2011). *Method for purifying 3,4-Dihydroxyphenylglycol (DHPG) from plant products.* (International Patent publication number WO/2010/070168).
Fernández-Bolaños, J. G., López, O., Fernández-Bolaños, J., & Rodríguez-Gutiérrrez, G. (2008). Hydroxytyrosol and derivatives: Isolation, synthesis, and biological properties. *Current Organic Chemistry, 12*, 442–463.
Gennadios, A., Hanna, M. A., & Kurth, L. B. (1997). Application of edible coatings on meats, poultry and Seafoods: A review. *LWT - Food Science and Technology, 30*(4), 337–350.
Gomez-Estaca, J., Bravo, L., Gómez-Guillén, M. C., Alemán, A., & Montero, P. (2009). Antioxidant properties of tuna-skin and bovine-hide gelatin films induced by the addition of oregano and rosemary extracts. *Food Chemistry, 112*, 18–25.
Kang, H. J., Jo, C., Kwon, J. H., Kim, J. H., Chung, H. J., & Byun, M. W. (2007). Effect of a pectin-based edible coating containing green tea powder on the quality of irradiated pork patty. *Food Control, 18*(5), 430–435.
Liu, L. S., Fishman, M. L., & Hicks, K. B. (2007). Composite films from pectin and fish skin gelatin or soybean flour protein. *Journal of Agricultural and Food Chemistry, 55*, 2349–2355.
Medina, I., Sacchi, R., Biondi, L., Aubourg, S. P., & Paolillo, L. (1998). Effect of packing media on the oxidation of canned tuna lipids. Antioxidant effectiveness of extra virgin olive oil. *Journal of Agricultural and Food Chemistry, 46*, 1150–1157.
Medina, I., Satué-Gracia, M. T., Bruce German, J., & Frankel, E. N. (1999). Comparison of natural polyphenol antioxidants from extra virgin olive oil with synthetic antioxidants in tuna lipids during thermal oxidation. *Journal of Agricultural and Food Chemistry, 47*, 4873–4879.
Nielsen, J. H., Sørensen, B., Skibsted, L. H., & Bertelsen, G. (1997). Oxidation in precooked minced pork as influenced by chill storage of raw muscle. *Meat Science, 46*(2), 191–197.
Nilsuwan, K., Benjakul, S., & Prodpran, T. (2016). Influence of palm oil and glycerol on properties of fish skin gelatin-based films. *Journal of Food Science and Technology, 53*(6), 2715–2724.
Oussalah, M., Caillet, S., Salmiéri, S., Saucier, L., & Lacroix, M. (2004). Antimicrobial and antioxidant effect of milk protein-based film containing essential oils for the preservation of whole beef muscle. *Journal of Agricultural and Food Chemistry, 52*, 5598–5605.
Pazos, M., Alonso, A., Sánchez, I., & Medina, I. (2008). Hydroxytyrosol prevents oxidative deterioration in foodstuffs rich in fish lipids. *Journal of Agricultural and Food Chemistry, 56*, 3334–3340.
Phisut, N., & Jiraporn, B. (2013). Characteristics and antioxidant activity of Maillard reaction products derived from chitosan-sugar solution. *International Food Research Journal, 20*, 1077–1085.
Rodríguez, G., Rodríguez, R., Fernández-Bolaños, J., Guillén, R., & Jiménez, A. (2007). Antioxidant activity of effluents during the purification of hydroxytyrosol and 3, 4 - dihydroxyphenyl glycol from olive oil waste. *European Food Research and Technology, 224*, 733–741.
Rodríguez-Gutiérrez, G., Duthie, G. G., Wood, S., Morrice, P., Nicol, F., Reid, M., ... de Roos, B. (2012). Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats-a proteomics and network analysis approach. *Molecular Nutrition & Food Research, 56*(7), 1131–1147.
Wegener, S., Bornik, M. A., & Kroh, L. W. (2015). D-Galacturonic acid: Highly reactive compound in nonenzymatic browning. 2. Formation of amino-specific degradtion products. *Journal of Agricultural and Food Chemistry, 63*, 6457–6465.
Witte, V. C., Krause, G. G., & Bailey, M. E. (1970). A new extraction method for determining 2-thiobarbituric acid values of pork and beef during storage. *Journal of Food Science, 35*(5), 582–585.
Zhou, P., Guo, M., Liu, D., Liu, X., & Labuz, T. P. (2013). Maillard-reaction-induced modification and aggregation of proteins and hardening of texture in protein bar model systems. *Journal of Food Science, 78*, C437–C444.

BLOQUE III

Título: Strawberry dietary fiber functionalized with phenolic antioxidants from olives. Interactions between polysaccharides and phenolic compounds[1].

Autores: Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, África Fernández-Prior, Blanca Vioque, Juan Fernández-Bolaños

Publicación: Food Chemistry

Título: Apple dietary fiber functionalized with phenolic antioxidants from olives. Interactions between polysaccharides and phenolic compounds[2].

Autores: Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, Heike Knicker, África Fernández-Prior, Juan Fernández-Bolaños

Publicación:

Resumen Bloque III

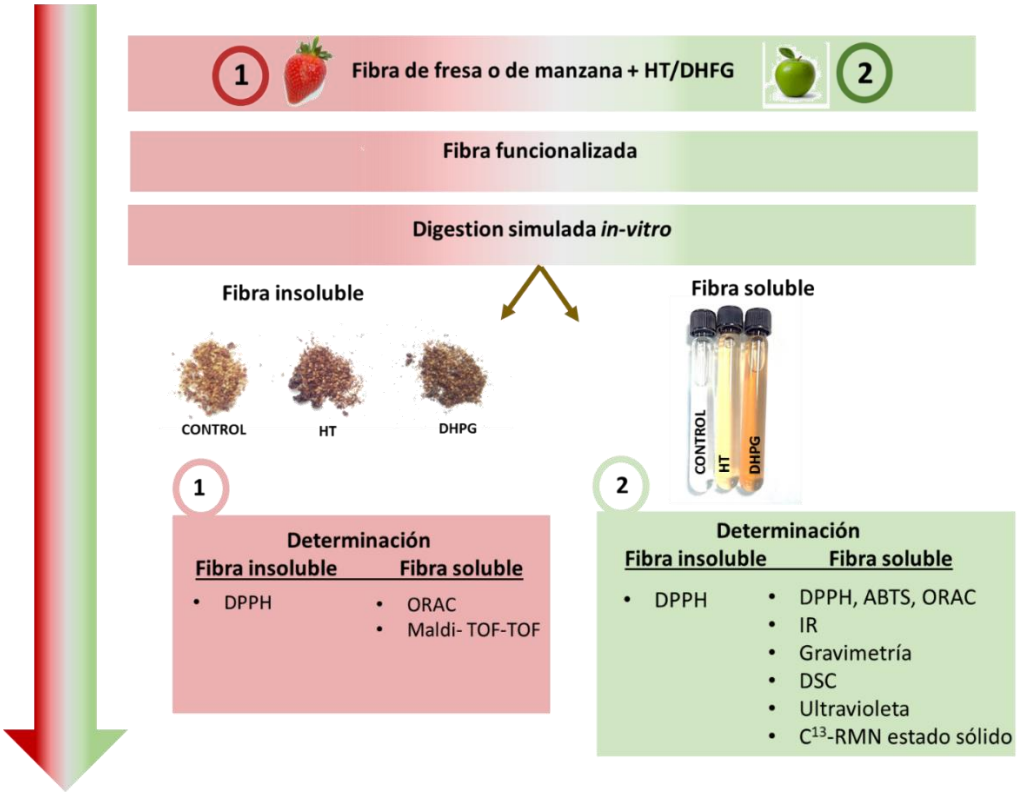
La fibra alimentaría es una parte esencial de nuestra dieta, y se caracteriza porque pasa el tracto intestinal sin ser digeridas por las enzimas humanas. Son solo parcialmente fermentados por las bacterias del colon pudiendo actuar como prebióticos. Existen dos tipos de fibra, la soluble y la insoluble. La fibra insoluble acelera el paso de los alimentos a través del estómago y los intestinos y le agrega volumen. Mientras que la fibra soluble retiene el agua y se vuelve gel durante la digestión, retarda la digestión y la absorción de nutrientes desde el estómago y el intestino.

Diversos estudios asocian una baja dieta en fibra con el aumento de la probabilidad de aparición de enfermedades crónicas y funcionales como el estreñimiento, enfermedad inflamatoria intestinal, apendicitis, síndrome del colon irritable y cáncer de colon. Por ello la OMS (Organización Mundial de la Salud) fija un consumo mínimo de fibra de 30 /persona /día, de la cual el 30% debe ser fibra soluble.

Así mismo desde hace más de una década se ha comenzado a introducir un nuevo concepto dentro de la fibra alimentaria y es “fibra alimentaria antioxidante”, la cual podría ser considerada como un potente ingrediente funcional. Es decir, si se añade un compuesto antioxidante a la fibra, se podría conseguir un efecto sinérgico debido a llegar a mejorar los efectos beneficiosos que ya presentan ambos compuestos de forma individual.

En este estudio se investigó la interacción de el hidroxitirosol (HT) y 3,4-dihidroxifenilglicol (DHFG), dos potentes antioxidantes fenólicos que se encuentran naturalmente en la aceituna con importantes propiedades biológicas, con la pared celular de fresa[1] y de manzana[2]. En ambos casos la interacción ocurre durante el proceso de secado de la pared celular. Después de digestiones simuladas *in vitro* fibras solubles e insolubles fueron obtenidas y estudiadas. La fibra obtenida después de las digestiones simuladas retenía una cantidad considerable de los compuestos antioxidantes. De hecho, estas propiedades

antioxidantes seguían presentes después de la formación del complejo HT/DHFG-fibra según ha sido determinado por ensayos de actividad antioxidante ORAC[1-2], DPPH[1-2], ABTS[2], análisis de MALDI-TOF-TOF[1] , análisis de calorimetría diferencial de barrido (DSC)2, ensayos termogravimétricos[2], IR[2], UV[2],redución del tamaño molecular mediante tratamiento enzimático observándose actividad antioxidante[1-2] , así como ensayos de C^{13}-RMN[2] en estado sólido, sugirieron la existencia del complejo fenol-fibra[2]. Además, en estos estudios se ha presentado una forma simple para la recuperación de fibra soluble funcionalizada con compuestos antioxidantes, HT/DHFG. La fibra que combina las propiedades funcionales de la fibra soluble e insoluble y de los compuestos antioxidantes podría ser un interesante complemento dietético para promover la salud intestinal.



Esquema resumen bloque III

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Strawberry dietary fiber functionalized with phenolic antioxidants from olives. Interactions between polysaccharides and phenolic compounds

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ABSTRACT

The interaction of strawberry cell wall with hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), two potent phenolic antioxidants naturally found in olive fruit with important biological properties, was investigated. The interaction occurred with drying and seemed to be more complex, strong and irreversible than a simple association. MALDI TOF-TOF analysis suggested covalent (ester bond) and non-covalent (strong hydrogen-bonding, mostly) interactions. The oxygen radical absorbance capacity (ORAC) assay confirmed that the phenols maintained partially their antioxidant activity after binding to the soluble dietary fraction. This soluble dietary fiber was obtained following digestion simulated *in vitro* with gastric and intestinal fluids. Although the antioxidant activity of HT and DHPG was affected by the dietary fiber interaction, this activity was restored when polysaccharide size was reduced by enzymatic treatment, suggesting that a similar process could occur in the colon. Thus, the use of this novel antioxidant-enriched soluble dietary fiber as a functional food ingredient could potentially promote intestinal health.

1. Introduction

Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) are the major antioxidant phenols found in olive fruit with potential health benefits that include antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic properties (Fernández-Bolaños, López, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2008). Furthermore, in 2012 the European Food Safety Authority (EFSA) endorsed the health claim that consumption of HT and its derivatives (≥5 mg/day) protects the cardiovascular system, preventing the oxidation of LDL cholesterol by free radicals (EFSA NDA Panel, 2012). This bioactivity is due to the molecular structure of both compounds that contains an *ortho*-diphenolic group and an additional hydroxyl group in the β position in the case of DHPG. These phenolic compounds are easily assimilated by the human body, reaching blood plasma in 15 or 20 min and eliminated 6–8 h later by the renal and digestive system (Echevarría, Ortiz, Valenzuela, & Videla, 2017). In previous studies we have demonstrated the potential binding interactions between pectin and HT or DHPG and described the formation of complexes as an efficient system for the delivery of the phenolic antioxidants to the colon. This encapsulation system protects the compounds from degradation during gastrointestinal transit to the colon (Bermúdez-Oria, Rodríguez-Gutiérrez, Rodríguez-Juan, González-Benjumea, & Fernández-Bolaños, 2018; Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017)

Interactions between intracellular polyphenols and plant cell walls have been described. Specifically, interactions between procyanidins and apple cell wall and individual components, cellulose, hemicelluloses and pectin have been shown, with the highest affinity found for interactions with pectin (Le Bourvellec & Renard, 2005). The polyphenols bind quickly and spontaneously to cell wall polysaccharides of dietary-fiber-rich foods when they are released by the rupture of fruits and vegetables during eating (grinding, mastication) or processing (boiling, autoclaving or freeze-drying) (Liu, Martinez-Sanz, Lopez-Sanchez, Gilbert, & Gidley, 2017). Therefore, the bioaccessibility of polyphenols would be affected by these interactions (Liu et al., 2017; Padayachee, Day, Howell, & Gidley, 2017). An important amount of ingested polyphenols is not bioaccessible after gastric and small intestinal digestion as it is bound to cell wall material (Saura-Calixto, 2011). These bound polyphenols reach the colon where they are released and fermented by bacterial microflora into absorbable metabolites that may be important for maintaining good gut health (Pozuelo et al., 2012). Furthermore, the phenolic compounds associated with

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soluble and insoluble dietary fiber can scavenge free radicals, protecting cells against oxidative damage in the intestinal ecosystem (Pérez-Jiménez & Saura-Calixto, 2015).

In previous works we observed that the simple phenolic compounds HT and DHPG form a strong interaction with soluble polysaccharides (pectin) via a combination of covalent (ester) and non-covalent bonds (hydrogen bonding and/or electrostatic interactions) as suggested by MALDI TOF-TOF analysis (Bermúdez-Oria et al., 2017, 2018). We also demonstrated that the phenols' antioxidant activity was maintained after complexation and after digestion simulated *in vitro* with gastric and intestinal fluids (Bermúdez-Oria et al., 2018).

The present study was carried out to obtain a potential source of dietary fiber from strawberry dietary fiber with higher antioxidant properties due to the addition of two potent phenolic compounds present in olive fruit, HT and DHPG. The aim was to identify free as well as complexed HT and DHPG in the soluble and insoluble dietary fiber of strawberry dietary fiber, to characterize putative interactions by MALDI TOF-TOF, and to determine the *in vitro* antioxidant activity of such complexes.

2. Materials and methods

2.1. Isolation of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from olive oil by-products

HT and DHPG were extracted by hydrothermal treatment at 50–80 °C for 60 min from *alperujo*, a by-product of olive oil extraction using the two-phase separation system. After treatment, a liquid phase rich in both compounds was obtained. HT and DHPG were purified by two different exchange chromatography system obtaining a 90–95% of purity in both compounds. These processes have been described and patented by Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, Fernández-Bolaños, Maya-Castilla, Rubio-Senent, and Marset Castro (2013).

2.2. Preparation of strawberry cell wall material

Alcohol-insoluble solids (AIS) from strawberry fruits were prepared according to the method of Renard (2005). Briefly, strawberries with the calix or green hull removed were cut into 2–3 pieces and directly grinded in a domestic blender in 70% ethanol and filtered on nylon cloth. The solid was ground and washed repeatedly with 70% ethanol until the filtrate became colorless. The final drying was performed by solvent exchange, 96% ethanol and acetone, and oven dried overnight at 40 °C.

2.3. Proximate composition of cell wall

Non-cellulosic sugar composition was determined by hydrolysis with 2 N trifluoroacetic acid (TFA) at 121 °C for 1 h. The released sugars were quantified after reduction and acetylation by gas chromatography (GC) (Englyst & Cummings, 1984). Cellulose was quantified from the glucose released after Saeman hydrolysis with sulfuric acid in two steps (Saeman, Moore, Mitchell, & Millett, 1954) and quantified as acetate of glucitol by gas chromatography, subtracting the glucose non-cellulosic material. Chromatographic conditions utilized were described by Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, and Fernández-Bolaños (2012). Klason lignin level was determined gravimetrically as the amount of acid-insoluble material remaining after sulfuric acid hydrolysis. Uronic acid was quantified using the phenyl-phenol method after sulfuric acid hydrolysis (Blumenkrantz & Asboe-Hansen, 1973).

2.4. Preparation of the strawberry dietary fiber-HT/DHPG complex

Approximately 0.5 g of strawberry dietary fiber alcohol insoluble solid was added to 10 mL of 1–10 mg/mL HT or DHPG solutions. After overnight swelling, the samples were dried by two methods: oven-drying for 72 h at 60 °C and 80 °C or freeze-drying (start temperature – 40–20 °C during 3 days).

2.5. Extraction and analysis of HT and DHPG from complex

Extraction of HT/DHPG present in the soluble and insoluble strawberry dietary fiber complex was evaluated in various organic solvents including methanol:water (20, 40, 80%), acetone, and dimethyl sulfoxide (DMSO):water (10, 30, 60, 90%) of a volume of 25 mL using 0.1 g dietary fiber. In addition, the extraction was assayed using the protocol for extraction of ester-linked phenol to fiber as described by Jaramillo et al. (2007). The sample was treated with 2 N NaOH for 24 h at room temperature under nitrogen. The solution was acidified and extracted three times with ethyl acetate. Also the complex was subjected to acid hydrolysis according to the method of Graciani and Vázquez (1980) for the quantification of HT from oleuropein (an olive molecule containing HT linked by ester bond to elenolic acid). 0.1 g of dry beads/emulsion were treated with 10 mL of 3 N HCl and heated to 100 °C for 10 min and filtered. The insoluble dietary fiber complex was subjected to the procedure of extraction of nonextractable polyphenols with strong acidic treatment, with methanol/H_2SO_4 90:10 (v/v) at 85 °C for 20 h, which releases hydrolysable polyphenols. An additional *n*-butanol/HCl 97.5:2.5 (v/v) treatment at 100 °C for 60 min was used to release anthocyanins (Pérez-Jiménez & Saura-Calixto, 2018). In no cases it was possible to quantify the strongly bound HT or DHPG.

The quantities of HT or DHPG were determined by HPLC according to a previously published method (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007).

2.6. Effect of the phenolic concentration on the formation of HT/DHPG-dietary fiber complex

The AIS material (0.5 g) was suspended in 10 mL of HT or DHPG solution to a concentration of 0.1, 0.5, 1.0, 1.5, 2.0, 5.0, 10, and 15 mg/mL. After soaking and hydration the mixture was oven-dried at 60 °C for 72 h. The solution of free phenol after re-hydration with water and 70% ethanol wash (200 mL) was separated from the dietary fiber material/phenol complexes by filtration under vacuum. The content of free HT or DHPG was measured by HPLC. The amount of phenolic compound bound to the dietary fiber was determined by subtracting the amount in the filtrate from that of the initial solution.

2.7. In vitro gastrointestinal digestion

2.7.1. Simulated gastric and intestinal fluids (process 1)

HT/DHPG-bound dietary fiber, free of soluble phenols, was immersed in 100 mL 0.1 M HCl solution at pH 1.2 (simulated gastric fluid) and incubated with gentle shaking in a water bath at 37 °C for the first 2 h. After incubation, the sample was filtered with filter paper, and the filtrate used for the quantification of HT and DHPH delivered in gastric fluid. The insoluble fraction was adjusted to pH 6.8 with the addition of 100 mL phosphate buffer solution (simulated intestinal fluid). The samples were incubated for another 3 h in a water bath at 37 °C with agitation. After incubation, the samples were filtered and the soluble fraction was separated from the insoluble fraction, and the HT/DHPG was quantified.

2.7.2. In vitro digestion with artificial gastrointestinal juices and enzymes (process 2)

Simulated *in vitro* gastric and intestinal digestion by addition of artificial gastrointestinal juices and gastrointestinal enzymes was carried out following the procedures described by Eprililati, D'Arcy, and Gidley (2009) and Padayachee et al. (2013) with slight modifications.

Simulated digestion – mouth

The pH of the sample (0.5 g HT/DHPG-dietary fiber) was adjusted to pH 6.9 with the addition of 5 mL diluted phosphate buffer containing α-amylase (1 g/L), 1.336 mM Ca_2Cl, 0.174 mM $MgSO_4$, 12.8 mM KH_2PO4, and 23.8 mM $NaHCO_3$ to simulate digestion in the mouth. The suspension was incubated at 37 °C for 5 min with continuous agitation.

Simulated digestion – stomach

The pH was readjusted to pH 2 with 5 mL of a solution of 5 mM KCl, 130 mM NaCl and 0.5 N HCl, which was added to the sample to simulate the lowest pH of the gastric environment. 0.5 mL pepsin solution (0.2 g pepsin from porcine gastric mucosa/5 mL 0.1 M HCl) was added and the mixture incubated for 1 h in a water bath at 37 °C with agitation.

Simulated digestion – intestine

The pH of the sample was gradually increased to pH 6–7 by the addition of 0.1 M $NaHCO_3$, followed by the addition of 2.5 mL pancreatin-bile salts solution (10 mg pancreatin from porcine pancreas and 50 mg porcine bile salts per 2.5 mL 0.1 M $NaHCO_3$). Sample was incubated for 2 h in a water bath at 37 °C with agitation. After incubation, the enzyme activity was terminated by the addition of 6 M HCl to pH 2, and the samples were centrifuged. The HT and DHPG content of the supernatant were quantified using HPLC.

2.8. Antioxidant activity measured by DPPH, ABTS and ORAC assays

2.8.1. Antioxidant activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

Antioxidant activity was measured using the DPPH method as described previously (Rodríguez et al., 2005). The radical-scavenging capacity of each antioxidant was expressed as percent $DPPH^{\cdot}$ remaining in solution after 30 min of reaction, calculated by the equation:

$$DPPH^{\cdot} \text{ remaining } (\%) = [(A_1 - A_2)/A_0] \times 100$$

where A_0 is the absorbance of the control, 100% of DPPH in methanol with water added instead of the sample, A_1 is the absorbance of the sample, and A_2 is the absorbance of blank sample, sample in methanol without $DPP\dot{H}$ reactive.

In the case of insoluble material, the antioxidant activity was evaluated as described Fuentes-Alventosa et al. (2009) with slight modifications. Briefly, 2–6 mg of insoluble material was suspended in 1 mL of $DPPH^{\cdot}$ reagent (3.8 mg/50 mL methanol). After 30 min of continuous agitation, the material was centrifuged, and the absorbance of the supernatant was measured at 490 nm.

2.8.2. Antioxidant activity, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

The ABTS assay was performed according to the method of Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, and Fernández-Bolaños (2012). This assay is based on the scavenging of ABTS radical ($ABTS^{\cdot +}$) by antioxidants. Results were expressed as percent $ABTS^{\cdot}$ remaining in solution, calculated by the equation:

$$ABTS^{\cdot} \text{ remaining } (\%) = [(A_1 - A_2)/A_0] \times 100$$

where A_0 is the absorbance of the control (water instead of the sample solution), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample under identical condition as A_1 with ethanol instead of $ABTS^{\cdot}$ solution.

2.8.3. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is based upon the inhibition of the peroxyl radical-induced oxidation initiated by thermal decomposition of 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH). The reactive oxygen species (ROS) generated from this thermal decomposition quenches the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples were assayed according to Ou, Hampsch-Woodill, and Prior (2001) with minor modifications. Sample was diluted with sodium phosphate buffer (10 mM, pH 7.4) and 25 μL of sample was transferred to a microplate. The blank well received 25 μL phosphate buffer while standards received 25 μL trolox solutions (10–140 μM). Then 150 μL of 1 μM fluorescein was added to all wells. After incubation (37 °C, 15 min), 25 μL AAPH (250 mM) was added to each well to initiate the reaction and a reading taken every 5 min for 90 min (Ex. 485 nm, Em. 538 nm) in a microplate reader (Fluoroskan Ascent™, Thermo Scientific™). Results were calculated using the difference of areas under the fluorescein decay curve between the blank and the sample and expressed as μmol Trolox equivalents/g of sample.

2.9. Enzymatic hydrolysis of HT/DHPG-bound soluble dietary fiber and ultrafiltration

The hydrolysis of soluble complex was carried out using a mixture of pectinolytic enzymes (4 μg/mL) including endo- and exo-polygalacturonase and pectin esterase (Novo Nordisk, Bagsvaerd, Denmark). The mixture (50 mL) was incubated at 37 °C for 24 h and the hydrolysis was terminated by heating to 100 °C for 10 min.

The hydrolyzed fraction was subjected to successive ultrafiltration using an Amicon 8400 stirred cell (Millipore Corporation, Bedford, MA, USA) through a molecular weight cut off of 5000, 3000, and 1000 Da. Each retained solution was washed with water until 300 mL of permeate was collected. Four fractions were obtained: the retained fractions > 5000 Da, > 3000 Da, and > 1000 Da, and the eluted fraction < 1000 Da. All fractions were analyzed for antioxidant activity by ORAC assay as above.

2.10. Characterization of HT/DHPG-dietary fiber complex by matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF-TOF)

The MALDI-TOF TOF mass spectra were acquired in positive ion mode over a mass-to-charge ratio (m/z) range of 150–2000 Da using an UltrafleXtreme Bruker mass spectrometer Smartbeam-II laser. The instrument was operated at an accelerating voltage of 26.45 kV with an extra voltage of 13.399 kV. Each spectrum was produced by accumulating data from 1000 to 2000 laser shots. The matrix solution of HCCA (alpha-cyano-4-hydroxycinnamic acid) was prepared in 10 mg/mL ACN:H_2O:TFA (50:47.5:2.5) (v/v/v) in the presence of sodium trifluoroacetate. The samples were desalted by dialysis during 72 h against deionized water in a dialysis tube of theoretical porosity of 12 kDa (Sigma-Aldrich, St Louis, MO, USA).

3. Results and discussion

3.1. Preparation of hydroxytyrosol (HT) and 3-4-dihydroxyphenylglycol (DHPG)-bound to strawberry dietary fiber

Components of dietary fiber (polysaccharides) have the ability to bind phenolic compounds (Liu et al., 2017; Saura-Calixto, 2011) and in some cases such complexes present an important antioxidant capacity (Pérez-Jiménez & Saura-Calixto, 2015; Wu et al., 2011). We suggested in previous studies that the natural phenols present in olive fruit, HT and DHPG, interact with pectin to form complex antioxidants (Bermúdez-Oria et al., 2017, 2018). In this work, we investigate if the interaction between these compounds with high antioxidant activity, and dietary fiber of strawberry or some components of the cell wall may occur. Such an interaction would possibly enhance the functional properties of dietary fiber by increasing its antioxidant activity.

Since fruit processing often involves tissue disruption and the polyphenols leave vacuolar organelles and bind quickly and spontaneously to the cell wall (Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012), we assayed the homogenization of strawberry fruits in the presence of HT and DHPG solutions (1 mg/mL) in a domestic

blender. However, after trying different contact times (1–24 h), the blended mixture was centrifuged and the totality of HT and DHPG was recovered in the supernatant. From an accidental discovery we observed that there was considerable retention when the mixture was fully dried at room temperature. Drying may cause irreversible modification to the cell wall with the collapse of the cell wall material (Le Bourvellec & Renard, 2005). It is also documented that boiling and drying decreases the binding affinity of apple cell walls for procyanidins due to pectin solubilization and degradation, and by altering the cell wall surface area (Le Bourvellec et al., 2012; Liu et al., 2017). However, drying seemed to enhance the binding of strawberry dietary fiber with HT/DHPG in our case.

To further evaluate the influence of drying on the cell wall's capacity to interact with HT and DHPG, cell wall material from strawberry was prepared as an alcohol insoluble solid. In addition, the influence of the main components of the cell wall, cellulose and pectin, were assayed. The cell wall material and their highly hydrophilic components were swollen in the presence of aqueous solutions of HT and DHPG (1 mg/mL) and then fully dried for 72 h at 60 °C and 80 °C in an oven or by lyophilization. After drying, the cell wall and their components were rehydrated with water and then washed copiously with ethanol 70%, and the dietary fiber/phenol complex separated by filtration. The content of free HT and DHPG was measured by HPLC and the bound phenols calculated by the difference (Fig. 1a). There was a slightly higher retention, statistically significant ($p < 0.05$), of HT to dried strawberry dietary fiber at 60° than 80 °C, but this was not the case for DHPG, where no significant difference between the two drying temperatures was observed. Cellulose and pectin also showed a high binding affinity for HT and DHPG. In the case of cellulose, DHPG was retained in a higher proportion when dried at 80 °C, while for pectin was no significant difference between the two drying temperatures. Freeze-drying showed a considerable retention for HT in cell wall while this retention was null in the case of DHPG. However, for cellulose and pectin, there were no significant differences between freeze- and oven-drying for HT, while DHPG presented lower retention following freeze-drying in the case of pectin. All further studies of the cell wall interaction with HT/DHPG employed oven drying at 60 °C.

The composition of the native strawberry cell wall material and the oven-dried (72 h at 60 °C) dietary fiber with phenol (HT) showed no significant differences (Table 1a). The two main sugars were glucose, essentially from cellulose and uronic acid from pectin, while other neutral sugars such as arabinose, xylose and galactose were also predominant, consistent with the sugar composition reported in the literature (Marlett & Vollendorf, 1994) (Table 1b).

3.2. Effect of concentration on phenolic adsorption

The effect of increasing the HT and DHPG concentration in the solution on the amount of phenols bound to the same amount of strawberry dietary fiber after drying was investigated. Fig. 1b shows the experimental, which represents the amount of bound HT or DHPG as a function of the free phenol concentration after re-hydration and 70% ethanol wash. For example, the amount of bound DHPG increased steadily at low concentrations but showed steeper increases of bound compound at higher concentrations until the amount of DHPG released was the same. The HT binding was similar although higher concentrations were required to see the steep increase at higher concentrations. However, as shown in the Fig. 1b, the results studied do not reach equilibrium.

3.3. Behavior of HT/DHPG-bound dietary fiber in digestive conditions

The HT/DHPG-bound dietary fiber was digested in two different *in vitro* simulated gastrointestinal media. In the first, the dietary fiber was digested in simulated gastric fluid (pH 1.2, 0.1 M HCl solution, 2 h) and next in simulated intestinal fluid (pH 6.8 phosphate buffer, 3 h). The dissolution of phenolic compound was monitored in each step. The second simulation of the digestion conditions of the gastrointestinal tract was carried out with the addition of digestive enzymes. The amount of phenols that remained bound to dietary fiber was determined for the final digestion process.

The samples of AIS (0.5 g) mixed with HT/DHPG solutions (1, 2, and 10 mg/mL) were fully dried for 72 h at 60 °C by oven- or freeze-dried and further re-hydrated and washed with ethanol (70%) to recover the unbound phenolic compound. The amount of HT and DHPG bound to the dietary fiber is presented in Fig. 2. For 1 mg/mL of initial bioactive compounds (BC) solution (20 mg/g AIS), 70% of HT and 40% of DHPG were bound to the dietary fiber when oven-dried and there was practically no release by gastric and intestinal conditions (Process 1, simulated gastric and intestinal fluids). An increase of retention of BCs was even observed using the enzymatic method (77% for HT and 66% for DHPG, Process 2), whereas the further dissolution of the phenolic compounds would be expected after the addition of digestive enzymes, resulting in enzymatic hydrolysis of protein, carbohydrates, and lipids. However, for simulated fluid digestion, while the retention of HT decreased to approximately 40% when the initial HT amount dispersed in the dietary fiber was increased to 40 and 200 mg/g AIS, the retention of DHPG remained fairly constant, at 40–50%. In the case of freeze-drying, the interactions between BC and dietary fiber were much weaker, with only 35% of HT bound to dietary fiber, half the amount following oven-drying, and no retention in the case of DHPG.

For the simulated gastric fluid, the binding interaction was very strong resulting in a minimal extraction of BC and practically no release was observed with the further pH change, simulating intestinal fluid (90–99% of total retention). In fact, even after the assay, extraction with organic solvent or alkaline and acid hydrolysis in severe conditions resulted in no extraction of HT and DHPG from the strongly bound dietary fiber (data not shown).

Dissolved fraction of phosphate buffer was separated from the insoluble fraction, with both fractions showing a high retention of HT and DHPG, confirmed by the color that comes from the initial compounds which was retained in both fractions (Fig. 2). This means that a high amount of the phenolic antioxidants in the soluble and insoluble fraction may be protected from absorption during gastrointestinal transit and this may reach the colon in significant amounts for fermentation by gut bacteria.

3.4. Antioxidant activity of the insoluble and soluble fractions

The antioxidant activity of the soluble and insoluble dietary fiber fraction linked to HT and DHPG was studied by the DPPH method in comparison with the corresponding soluble and insoluble fraction (control) obtained from the dietary fiber of strawberry but without the addition of BCs. The results obtained for the insoluble fraction of 40 mg BC/g AIS and 200 mg BC/g AIS complexes are presented in Fig. 3. The assayed fibers were phenol-free, so their antioxidant activity is exclusively due to that corresponding to the fiber fraction or complexed BC. Indeed, no differences were found between the samples with HT or DHPG added and the control. This is indicative that strawberry fiber has an important amount of phenol linked to fiber (0.44 g/100 g dry matter for a total of dietary fiber of 24.9/100 g dry matter), as has been reported to contribute to the fiber fraction's antioxidant activity (Saura-Calixto, 2011). These compounds are called non-extractable polyphenols or macromolecular antioxidants, and include fundamentally proanthocyanidins and hydrolysable tannins, which remain in the insoluble dietary fiber of strawberry and reach the colon with promising results in relation to gastrointestinal health (Pérez-Jiménez & Saura-Calixto, 2015). However, the HT and DHPG linked to the insoluble fraction did not maintain their antioxidant properties.

The antioxidant activity of the soluble fraction was studied by three different methods (DPPH, ABTS and ORAC assays) and the results for two concentrations of HT and DHPG added to the mixture with the AIS

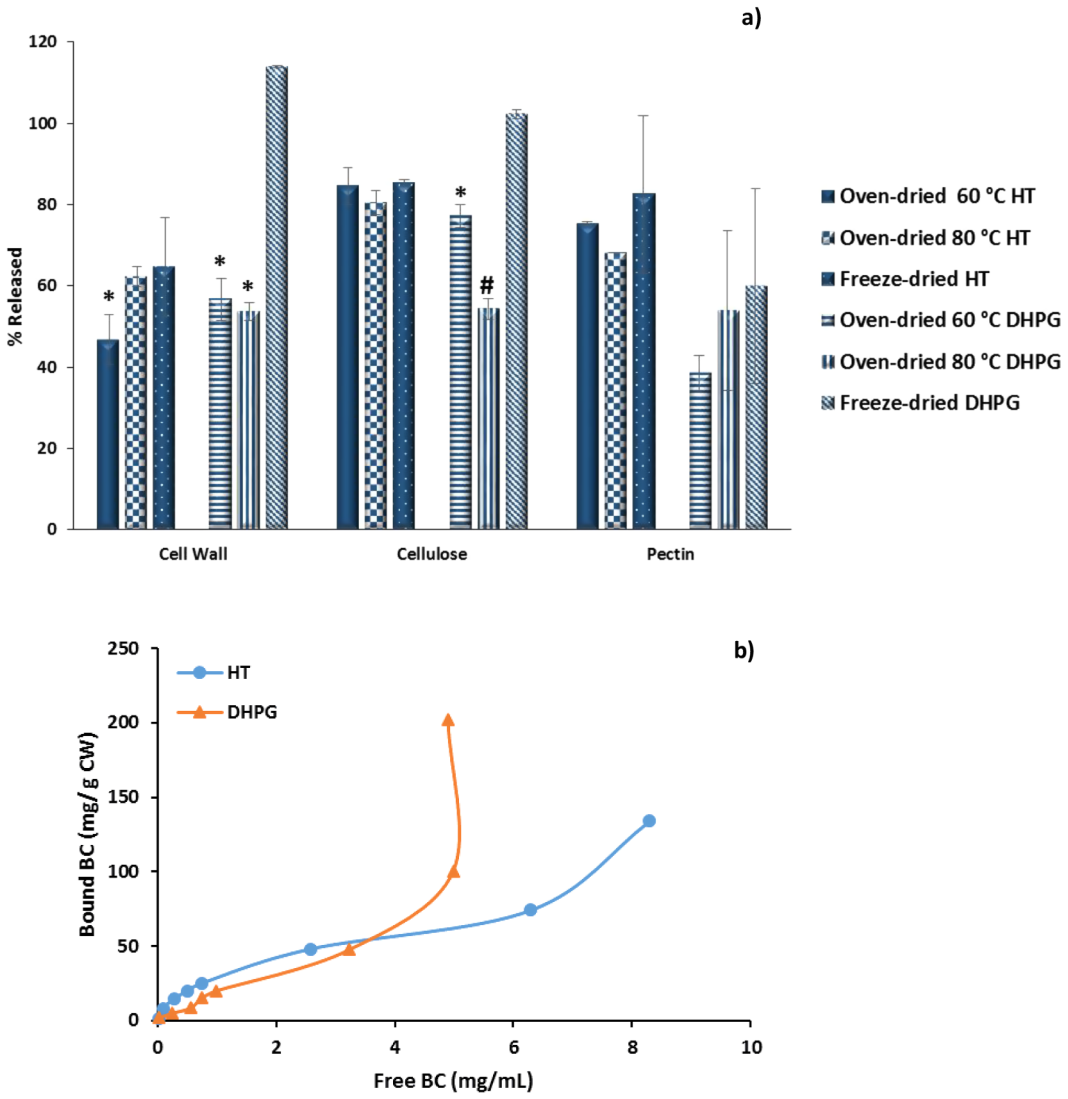


Fig. 1. a) Percentage of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) released after interaction with strawberry cell wall, cellulose and pectin following different drying conditions (60 °C and 80 °C oven-drying or freeze-drying). Initial concentration of bioactive compounds (BCs) 1 mg/mL; cell wall (CW), cellulose and pectin 50 mg/ml. Error bars represent standard deviations (n = 3). $^{*}p < 0.05$ and $^{\#}p < 0.05$ indicate significant differences between drying conditions for the bioactive compound and the matrix. b) Effect of the bioactive compounds (BC) (0.1, 0.5, 1.0, 1.5, 2.0, 5.0, 10, and 15 mg/mL) on the formation of HT/DHPG-dietary fiber complex of strawberry.

(40 and 200 mg/g AIS) are presented in Fig. 4a and b. In the case of the lower concentration (40 mg/g AIS), no differences were found between BC-bound soluble dietary fiber and the control for both the DPPH and ABTS assays. However, for the sample of soluble fraction with a higher concentration (200 mg/g AIS) of linked-DHPG, more free radical scavenging activity was observed for both methods. This confirmed that the DHPG linked to soluble dietary fiber maintains some antioxidant activity. It is possible that the additional −OH group of DHPG with respect to HT helps to provide greater availability of the catechol group, which is responsible for the BCs' antioxidant activity, a result that is in agreement with a previous report on DHPG-pectin complex formation via encapsulation (Bermúdez-Oria et al., 2018).

The ORAC determinations revealed significant differences between HT and the control as well as DHPG and the control, with a particularly significant increase in activity for the higher concentrations of BC in the complex (Fig. 4c). However, with this assay, no differences were observed between HT and DHPG, which does not support the hypothesis that the hydroxyl group in the ortho position of DHPG allows the compound to act as an antioxidant in contrast to HT. Nevertheless, this finding is consistent with a previous study, which reported an interaction between polyphenols from tea and β-glucan, and displayed oxygen scavenging-activity indicative of the complex's antioxidant behavior (Wu et al., 2011).

Since the soluble dietary fiber control did not present antioxidant activity or was very low, whereas the insoluble fiber control did, we can conclude that very little polyphenols were associated to the soluble dietary fiber of strawberry, although there has been a report of the presence of a small fraction of flavonoids and phenolic acid (Saura-Calixto, 2011). Nevertheless, the two potent phenolic antioxidants HT and DHPG form a strong complex with the soluble polysaccharides of strawberry dietary fiber and impart their antioxidant properties to the complex.

The finding of soluble polysaccharides with antioxidant activity (BC-bound soluble dietary fiber) that are indigestible in the upper gastrointestinal tract but fermented in the large intestine make this complex an interesting putative dietary supplement because it may promote intestinal health. The combination of the properties of both components would provide a single material capable of scavenging free

Table 1
a) Composition (mg/g cell wall) of native cell wall material (AIS) and the oven-dried cell wall with HT added (HT-cell wall dried, 60 °C/72 h) isolated from strawberries by the alcohol insoluble solid method. Each value is the average of three replicates ± SD. b) Composition (mg/g cell wall) of the HT/DHPG-soluble dietary fiber complex. Each value is the average of four replicates using two HT and DHPG-soluble fiber complex samples. Percentage molar of uronic acid and neutral non-cellulosic sugars are listed.

a)	mg/g cell wall	
	Native cell wall	HT-cell wall dried
Cellulose	104.0 ± 3.7	93.9 ± 4.1
Lignin	198.0 ± 8.1	256.4 ± 16.2
Uronic acid	149.1 ± 14.1	99.8 ± 15.1
Hemicellulose (non-cellulosic sugars)	100.3 ± 12.6	120.2 ± 7.2
Moisture	101.1 ± 5.4	98.4 ± 2.8
	% molar of non-cellulosic sugar	
Rhamnose	2.01 ± 0.43	2.22 ± 0.78
Fucose	1.15 ± 0.09	0.97 ± 0.38
Arabinose	10.23 ± 0.64	10.92 ± 0.95
Xylose	17.50 ± 2.72	16.83 ± 2.88
Mannose	1.37 ± 0.14	1.72 ± 0.45
Galactose	9.28 ± 0.95	10.71 ± 1.52
Glucose	2.69 ± 0.46	2.95 ± 0.85
Uronic acid	55.76 ± 4.71	40.28 ± 10.33

b)	HT/DHPG-soluble dietary fiber complex (mg/g cell wall)
Uronic acid	115.7 ± 6.4
Neutral sugar	84.6 ± 12.9
	% molar
Rhamnose	1.48 ± 0.73
Fucose	0.16 ± 0.06
Arabinose	5.56 ± 1.00
Xylose	0.98 ± 0.25
Mannose	0.25 ± 0.03
Galactose	4.24 ± 0.98
Glucose	1.10 ± 0.48
Uronic acid	90.36 ± 4.99

radicals and counteracting the effect of dietary pro-oxidants, as well as the potential beneficial systemic effects due to generation of short chain fatty acids (acetic, propionic, butyric) by fermentative microflora (Saura-Calixto, 2011).

3.5. Soluble dietary fiber composition

Based on monosaccharide analysis of the soluble fraction and its uronic acid content (Table 1b), pectin was observed to be the predominant component of the HT/DHPG-bound complex. This is in agreement with our previous reports where the strong binding of HT and DHPG with pectinate beads was reported (Bermúdez-Oria et al., 2017, 2018). Therefore, soluble fibers such as pectin with associated antioxidant compounds could be of interest to the food industry due to its health benefits (Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006) and technological applications (Thakur, Singh, Handa, & Rao, 1997).

3.6. Release of pectin fragments: Simulation of hydrolysis in the colon

A mixture of pectinolytic enzymes was added to reduce the molecular size of the HT/DHPG-soluble dietary fiber complex in order to provide information about the effect that pectinase, produced by the colonic microflora, may have on the antioxidant properties of the complex. The solubilized fraction after enzymatic digestion was subjected to sequential ultra-filtration through a 5000, 3000, and 1000 Da molecular weight cut-off membrane. The corresponding eluted and retained fractions were analyzed for antioxidant activity by the ORAC assay (Fig. 4d). The antioxidant activity of the fraction below 1000 Da was similar to that obtained above 5000 Da in the case of the complex with HT. For the DHPG-bound complex, the activity appeared relatively high in all fractions. In addition, the antioxidant activity of both fractions > 5000 Da were similar to the activity of the initial fractions of HT/DHPG linked to soluble dietary fiber (Fig. 4c). Together these results indicate that although the antiradical activities of HT and DHPG seem to be directly or indirectly affected by their interaction with polysaccharides, mostly pectin, their antioxidant activity is restored when the size of the polysaccharides is reduced. The reduction in size of the oligomers or the actual hydrolytic process of soluble dietary fiber or pectin by colonic bacterial enzymes could differ to the outcomes obtained in this study; however, this result suggests that the hydrolytic process in the colon releases oligomers with potential antioxidant activity. This activity could help to prevent certain kinds of degenerative or chronic diseases such as colon cancer or inflammatory bowel disease (IBD) (Saura-Calixto, 2011), although future research is needed to verify this hypothesis.

3.7. Investigation of the interaction between HT/DHPG and the soluble fraction

The presence of a complex between the soluble dietary fiber fraction and HT/DHPG, which is not hydrolysable, even by the most severe methods applied to non-extractable polyphenols or macromolecular antioxidants(Pérez-Jiménez & Saura-Calixto, 2015), and gives no detectable UV absorption signal (data not shown), was confirmed by MALDI TOF-TOF analysis.

The MALDI TOF-TOF mass spectra of HT and DHPG-bound to the soluble fraction in positive ion mode (Fig. 5A and B) showed a diversity of masses that was not present in the corresponding control without phenolic compound (data not shown). After studying possible combinations of different oligosaccharides with galacturonic acid units (194 Da), including methyl-ester groups (208 Da), pentoses (150 Da), hexoses (180 Da), and a fragment described by Domon and Costello (1988) that corresponds to a cross-ring of 60 Da from the non-reducing end, 134 Da if containing the reducing end of a galacturonic acid-type, or 120 Da of a hexose, and the corresponding possible adduct of sodium, did not found values *m/z* without phenol. It is important to remember that sugars (including uronic acid) lose 18 Da ($-H_2O$) per glycosidic bond. In all cases the addition of one or two molecules of HT (154 Da) or DHPG (170 Da) was necessary to obtain coincidence with *m/z* values of the HT/DHPG-oligosaccharide complex. Several tentative structures taking into account all the possible combinations are proposed in Fig. 5A and B. However, unequivocal evidence of non-covalent interactions (electrostatic and dipolar forces, hydrogen binding, hydrophobic interactions and/or van der Waals attractions) between HT/DHPG with soluble dietary fiber is difficult to find. Similarly, finding evidence to support the formation of covalent bonds between oligosaccharides of galacturonic acid and HT or DHPG as an ester that would explain the irreversible complexes that maintain in part the antioxidant activity of the BCs has proven very difficult.

The development of strong H-bonds between polyphenol derived from tea and β-glucan (Wu et al., 2011) or strong hydrophobic H-bonds between polysaccharides, mainly pectin, and polyphenols during wine making (Renard, Watrelot, & Le Bourvellec, 2017) have been reported in the literature. Furthermore, staining colorant, formed mainly by purple-blue anthocyanins and used during centuries as a natural cotton fabric dye, have been hypothesized to form H-bonds with cellulose fibers (Saura-Calixto, 2011). Also, more recently, electrostatic interactions were shown to be important in the interaction between polyphenols and cellulose-based composite and apple cell walls (Phan, Flanagan, D'Arcy, & Gidley, 2017). However, clear evidence of a HT/DHPG-soluble dietary fiber binding mechanism is still lacking. The formation of irreversible complexes between polyphenols and

Simulated fluid digestion

1º Step

- 500 mg AIS with BC
- Driying process 60 °C/72h

2º Step

- Extraction with 200 mL EtOH

3º Step: Gastric Fluid

- pH= 1.2 , 0.1 M HCL / 2h

4º Step: Intestinal Fluid

- pH= 6.8, Phospahte Buffer (PB) / 3h

Process 1

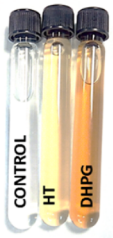
	Initial BC (mg)		Released EtOH	% Bound	Released HCl (mg)	Released PB (mg)	% Bound
Oven-dried 60°C	10	HT	2.78 ± 0.96	69.85 ±9.40	0.24 ± 0.02	n.d	96.74 ± 0.14
		DHPG	5.67 ± 1.24	39.65 ±14.35	0.37 ± 0.20	0.04 ± 0.01	89.56 ± 7.71
	20	HT	7.48 ± 0.87	61.81 ±4.28	0.16 ± 0.01	n.d	98.69 ± 0.01
		DHPG	9.95 ± 1.01	49.90 ±4.78	0.07 ± 0.06	n.d	99.28 ± 0.48
	100	HT	57.92 ± 8.98	40.06 ±10.05	2.03 ± 1.07	0.22 ± 0.04	94.27 ± 3.84
		DHPG	49.84 ± 12.17	47.33 ±13.09	2.84 ± 0.92	0.45 ± 0.01	93.02 ± 3.50
Freeze-dried	10	HT	6.46 ± 0.22	35.38 ±2.25	0.06 ± 0.00	n.d	98.37 ± 0.01
		DHPG	10	0	-	-	-

Phosphate buffer

Insoluble Fraction (340 mg)*

- DPPH

Soluble Fraction (160 mg)*



- DPPH
- ABTS
- ORAC
- MALDI TOF-TOF

Simulated fluid and enzymatic digestion

1º Step

- 500 mg AIS with BC
- Driying process 60 °C/72h

Process 2

2º Step: Mouth

- 500 mg CW, α-amilase 1g/L
- 37 ° C /5 min

3º Step: Stomach

- 0.5 ml pepsin 40mg/mL , 37° C / 1h
- pH= 6-7 with $NaHCO_3$ 0.5M

4º Step: Intestine

- 10 mg pancreatin and 50 mg porcine bile in 2.5 ml $NaHCO_3$ 0.1M
- 37° C / 2h
- pH= 2 with HCl 0.5N.

Initial BC (mg)		Released BC (mg)	% Bound
10	HT	2.27 ± 0.16	77.3 ± 1.56
	DHPG	3.43 ± 0.06	65.7 ± 0.57

Fig. 2. Scheme of the two *in vitro* simulated gastrointestinal digestion process of HT/DHPH binding to strawberry cell wall. Percentage of bioactive compounds (BC) bound to AIS during drying process (oven-dried and freeze-dried) and bound in the final simulation digestion process respect to the BC bounded. *Represent the average weight of the insoluble and soluble fraction obtained from digestion process of HT-bound cell wall for an initial concentration of HT of 2 mg/ml (10 mL of solution on 500 mg of cell wall and oven-dried at 60 °C for 72 h) (n = 3) BC: bioactive compound; BP: phosphate buffer; AIS: alcohol insoluble solid.

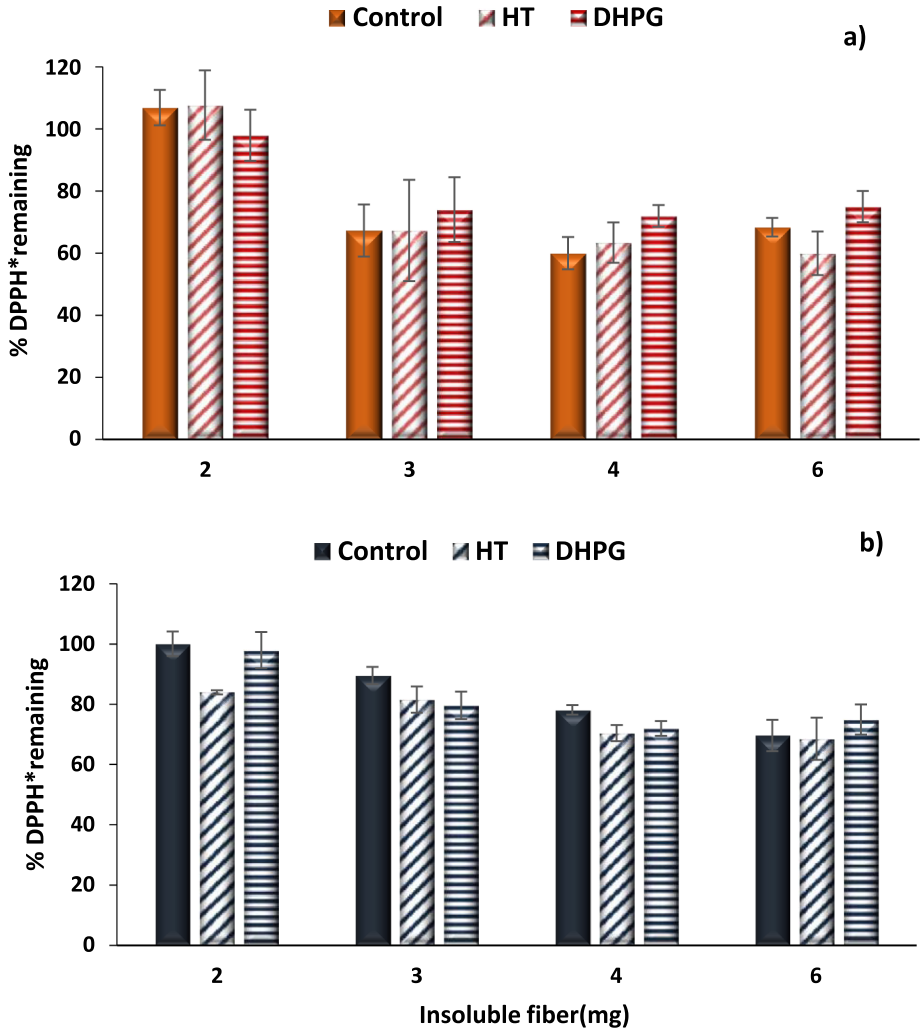


Fig. 3. Antiradical capacity of insoluble fiber (2, 3, 4 or 6 mg) bound to HT/DHPG obtained from an initial bioactive compound (BC) concentration of (a) 2 mg/mL (40 mg BC/g CW) (b) 10 mg/mL (200 mg BC/g CW). Comparison with insoluble dietary fiber without BC added (Control). Antiradical capacity is expressed as percentage DPPH$^{\cdot}$ remaining in solution after 30 min of reaction. Each bar is the average value of three replicates. Error bars represent standard deviations (n = 3). CW: cell wall.

polysaccharides are frequently observed in the formation of pomace during juice extraction. Covalent interactions may be mediated by the oxidation of procyanidins, with the subsequent formation of highly electrophilic *ortho*-quinones mediated by polyphenoloxidase, which can lead to the formation of covalent bonds with macromolecules (Renard et al., 2017). In our case, the antioxidant activity found in the complex excludes the complete oxidation of *ortho*-diphenols to quinone and the formation of such a covalent interaction. For the signals at *m/z* 895 there are two possibilities of non-covalent or covalent binding of HT (Fig. 5A). The mass difference of 160 *m/z* units between the signals 895, 1057 and 1219 corresponds to the loss of hexose units, probably galactose. Also for *m/z* 1026, two possibilities were found for the same signal, with a combination of covalent and non-covalent in both possibilities. For DHPG, the ions at *m/z* 833 were attributed to a combination of tetramers bound to DHPG by an ester bond and by non-covalent bonds (Fig. 5B). The mass difference between the signals 833, 995, 1157, 1319, 1481, and 1643, were 160 *m/z* units corresponding to the loss of hexoses, probably galactose, from a nonamer.

Although the possibility of covalent bonds, such as ester bonds, was found for HT and DHPG by MALDI TOF-TOF mass spectroscopy analysis, the release of these compounds by acid or alkali hydrolysis was not achieved. Yet alkaline hydrolysis generally hydrolyzes ferulic acids ester bonds when applied to cereal products or sugar-beet cell walls (Ralet, Thibault, Faulds, & Williamson, 1994). Also, the formation of covalent adducts between cell-wall and procyanidin due to the impact of thermal treatment has been recently described. During canning of pear piece, a *neo*-formed pink color was produced, which was not re-extractable with solvent or with enzymes and persisted in the residues, leading to the suspected formation of covalent adducts (Le Bourvellec et al., 2013). In our case, due to the effect of drying, we presume that a combination of non-covalent and covalent bonds, probably in the form of covalent adducts, were responsible for the strong binding of HT/DHPG to the dietary fiber.

4. Conclusions

Interaction between HT and DHPG, two natural phenols present in olive fruit, and the dietary fiber of strawberry fruits occurred after drying but did not occur after contact between suspended cell wall and phenols in solution. The phenolic compounds also bound to commercial pectin and cellulose after drying. The binding seemed very strong and non-reversible. From MALDI TOF-TOF analysis we suggested that this

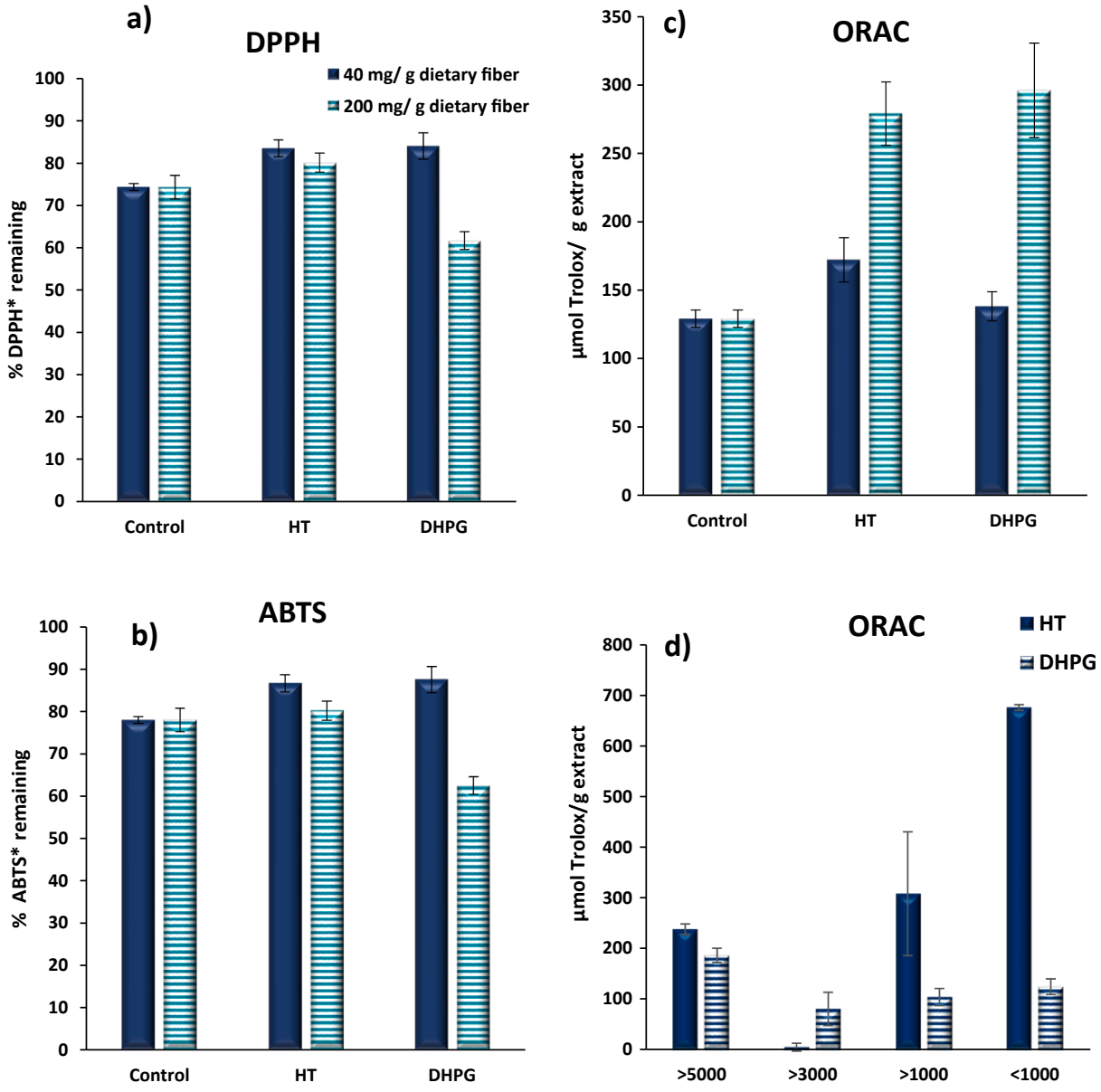


Fig. 4. Antiradical capacity of the HT/DHPG-soluble dietary fiber complex obtained from two different initial concentrations of bioactive compound (BC), 2 mg/mL (40 mg of BC/g CW) and 10 mg/mL (200 mg BC/g CW). Comparison with soluble dietary fiber without BC added (Control). a) Antiradical capacity is expressed as percent DPPH* remaining. b) Antiradical capacity is expressed as percent ABTS* remaining. c) Oxygen radical capacity (ORAC) is expressed as µmol Trolox/g extract. Each bar is the average value of three replicates. d) Antioxidant activity measured by ORAC of recovered fractions after a sequential ultrafiltration process (5000, 3000 and 1000 Da) obtained from HT/DHPG-soluble dietary fiber complex treated with a mixture of pectinolytic enzymes. Results are expressed as µmol Trolox/g extract. Error bars represent standard deviations (n = 3).

strong interaction was due to a combination of covalent (ester bond) and non-covalent bonds (hydrogen bonding, electrostatic interaction). Further work to isolate and characterize the nature of these bonds is in progress. The effect of simulated gastric and small intestinal digestion on HT/DHPG-bound to dietary fiber material was also examined. The dissolved fraction of phosphate buffer was separated from the insoluble fraction and both fractions showed a high retention of HT and DHPG, confirmed by the color imparted by the initial compounds and retained in both fractions. Moreover, HT/DHPG-soluble and insoluble dietary fiber complexes showed antioxidant activity. Therefore, a high amount of antioxidants in the soluble and insoluble dietary fiber fraction may be protected from absorption during gastrointestinal transit to reach the colon. However, the potent phenolic antioxidants HT and DHPG only retained antioxidant properties after forming a strong complex with the soluble polysaccharides of strawberry dietary fiber. Interestingly, when the size of the polysaccharide was reduced by enzymatic treatment, the antioxidant activity of HT/DHPG affected by the interaction with components of cell wall was restored, which suggests that a similar process could occur in the colon. From a technological point of view, we present a simple method for the recovery of soluble dietary fiber supplemented or enriched with antioxidant compounds. The use of this new fiber combining the functional properties of both soluble dietary fiber and antioxidant could to be an interesting dietary supplement to promote intestinal health. Future research on the health benefits of dietary fiber linked to HT and DHPH are necessary to attest their functionality (Granato et al., 2018).

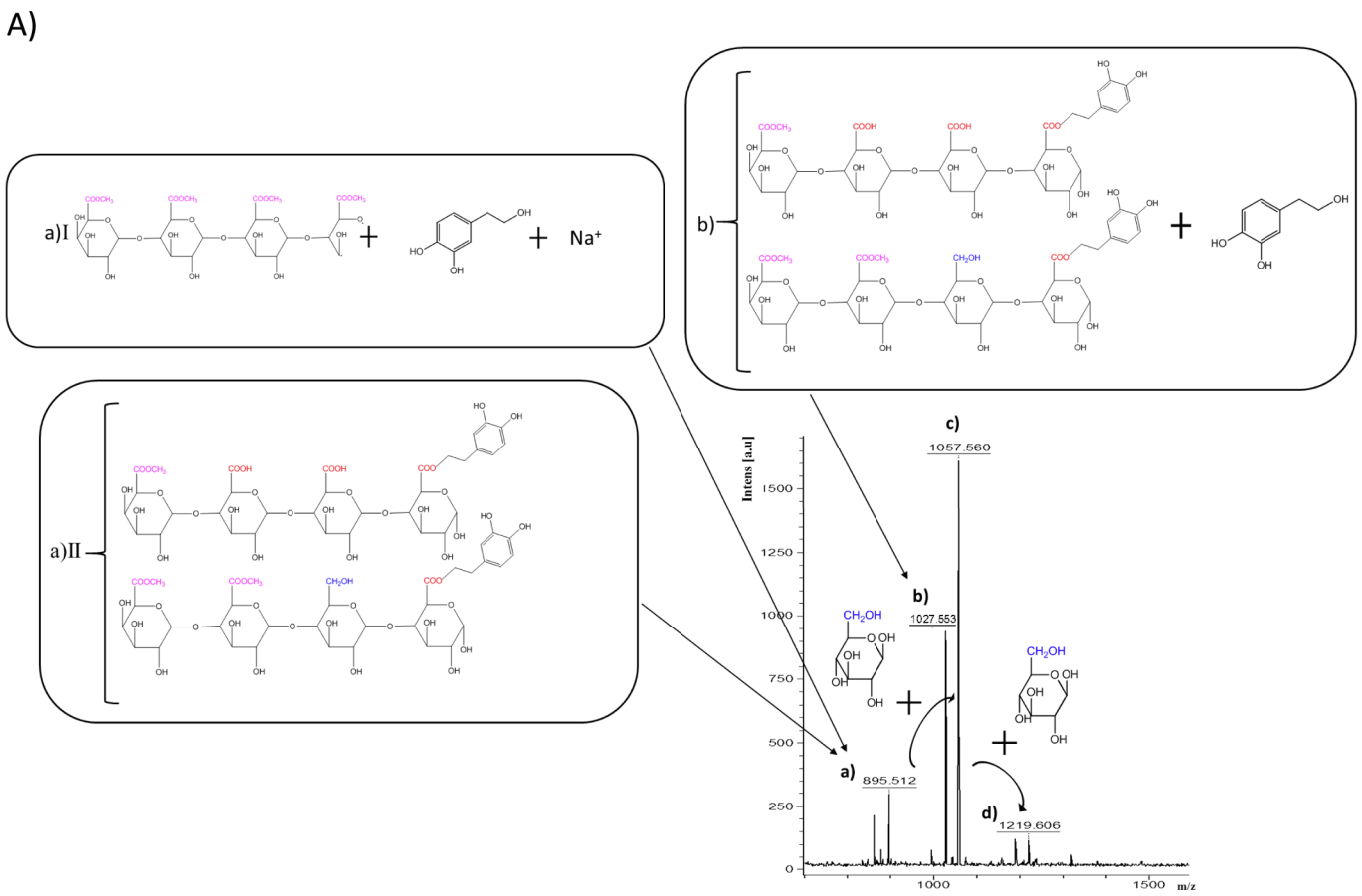


Fig. 5. MALDI-TOF TOF mass spectra (positive mode) of dialyzed HT/DHPG-soluble dietary fiber complex. A) Tentative structures of the *m/z* signal at 895 and 1027 for HT complex. B) Tentative structures of the signal *m/z* at 833 for DHPG complex. The mass difference between other signals was of 162 *m/z* units that may correspond to hexose moieties.

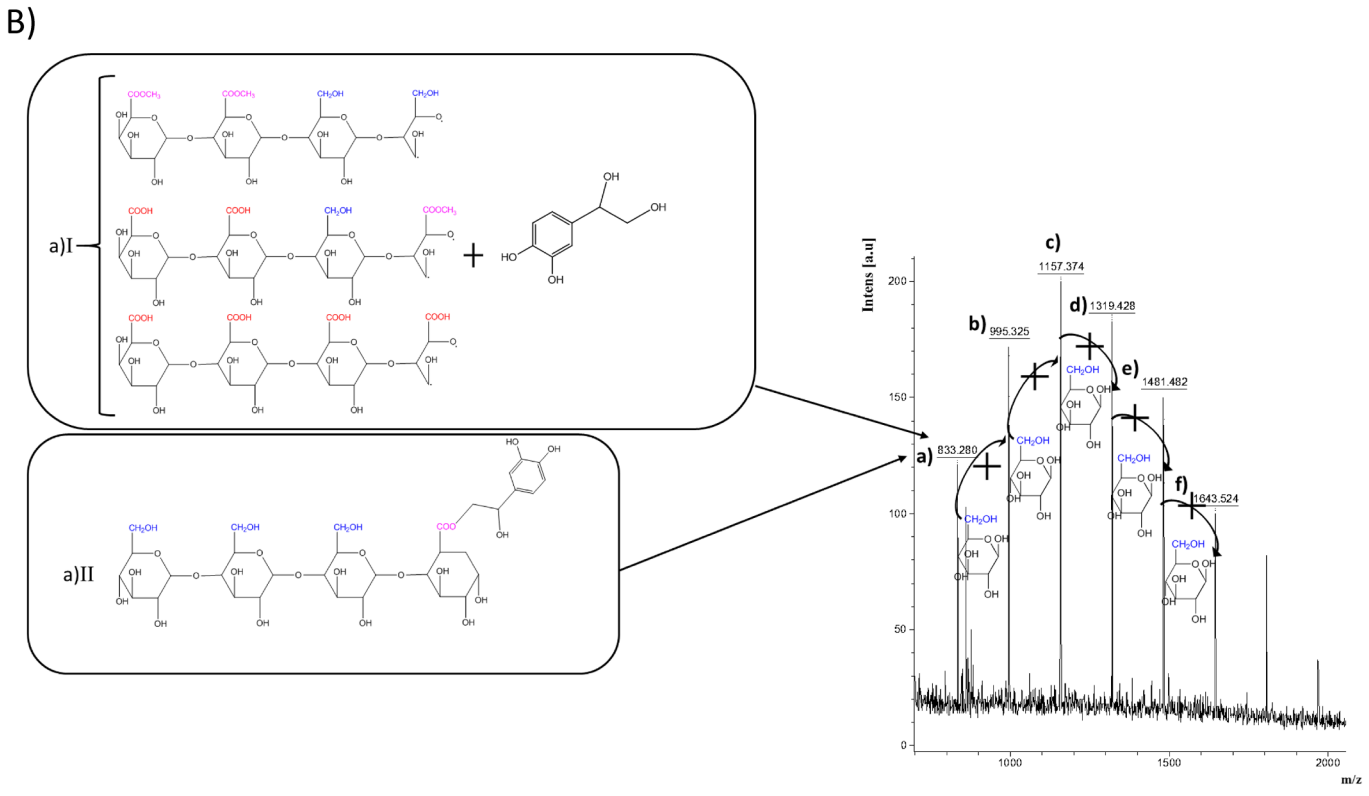


Fig. 5. (*continued*)

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rodríguez-Juan, E., González-Benjumea, A., & Fernández-Bolaños, J. (2018). Molecular interactions between 3,4-dihydroxyphenylglycol and pectin and antioxidant capacity of this complex in vitro. *Carbohydrate Polymers, 197*, 260–268. https://doi.org/10.1016/j.carbpol.2018.05.089.

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Lama-Muñoz, A., & Fernández-Bolaños, J. (2017). Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting. *Carbohydrate Polymers, 163*, 292–300. https://doi.org/10.1016/j.carbpol.2017.01.027.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*(2), 484–489.

Domon, B., & Costello, C. E. (1988). Structure elucidation of glycosphingolipids and gangliosides using high-performance tandem mass spectrometry. *Biochemistry, 27*(5), 1534–1543. https://doi.org/10.1021/bi00405a021.

Echevarría, F., Ortiz, M., Valenzuela, R., & Videla, L. A. (2017). Hydroxytyrosol and cytoprotection: A projection for clinical interventions. *International Journal of Molecules Science, 18*, 930–944. https://doi.org/10.3390/ijms18050930.

Efsa, NDA Panel (2012). Scientific Opinion on the substantiation of a health claim related to polyphenols in olive and maintenance of normal blood HDL cholesterol concentrations (ID 1639, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal, 10*, 2848. https://doi.org/10.2903/j.efsa.2012.2848.

Englyst, H. N., & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *The Analyst, 109*(7), 937. https://doi.org/10.1039/an9840900937.

Eprililati, I., D'Arcy, B., & Gidley, M. (2009). Nutriomic analysis of fresh and processed fruit products. 1. During in vitro digestions. *Journal of Agricultural and Food Chemistry, 57*, 3363–3376. https://doi.org/10.1021/jf900368p.

Fernández-Bolaños, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J. M., Maya-Castilla, I., Rubio-Senent, F., ... Marset Castro, A. (2013). Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroxyphenylglycol extract, and hydroxytyrosyl acetate extract from by-products of the olive tree and the purification of thereof. International Patent No. WO 2013/007850A1.

Fernández-Bolaños, J. G., López, Ó., Fernández-Bolaños, J., & Rodríguez-Gutiérrez, G. (2008). Hydroxytyrosol and derivatives: Isolation, synthesis, and biological properties. *Current Organic Chemistry, 12*(6), 442–463. https://doi.org/10.2174/138527208784083888.

Fuentes-Alventosa, J. M., Rodríguez-Gutiérrez, G., Jaramillo-Carmona, S., Espejo-Calvo, J. A., Rodríguez-Arcos, R., Fernández-Bolaños, J., ... Jiménez-Araujo, A. (2009). Effect of extraction method on chemical composition and functional characteristics of high dietary fibre powders obtained from asparagus by-products. *Food Chemistry, 113*(2), https://doi.org/10.1016/j.foodchem.2008.07.075.

Graciani, E., & Vázquez, A. (1980). Estudio de los componentes del aceite de oliva por cromatografía líquida de alta eficacia (HPLC). II Cromatografía en fase inversa. *Grasas y Aceites, 31*, 237–243.

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L. D., Hidalgo, F. J., ... Finglas, P. (2018). Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? *Food Chemistry, 264*, 471–475. https://doi.org/10.1016/j.foodchem.2018.04.012.

Jaramillo, S., Rodríguez, R., Jiménez, A., Guillén, R., Fernández-Bolaños, J., & Heredia, A. (2007). Effects of storage conditions on the accumulation of ferulic acid derivatives in white asparagus cell walls. *Journal of the Science of Food and Agriculture, 87*(2), 286–296. https://doi.org/10.1002/jsfa.2718.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids, 28*(1), 92–104. https://doi.org/10.1016/j.foodhyd.2011.11.008.

Le Bourvellec, C., Gouble, B., Bureau, S., Loonis, M., Plé, Y., & Renard, C. M. G. C. (2013). Pink discoloration of canned pears: Role of procyanidin chemical depolymerization and procyanidin/cell wall interactions. *Journal of Agricultural and Food Chemistry, 61*(27), 6679–6692. https://doi.org/10.1021/jf4005548.

Le Bourvellec, C., & Renard, C. M. G. C. (2005). Non-covalent interaction between procyanidins and apple cell wall material. Part II: Quantification and impact of cell wall drying. *Biochimica et Biophysica Acta, 1725*(1), 1–9. https://doi.org/10.1016/j.bbagen.2005.06.003.

Le Bourvellec, C., Watrelot, A. A., Ginies, C., Imberty, A., & Renard, C. M. G. C. (2012). Impact of processing on the noncovalent interactions between procyanidin and apple cell wall. *Journal of Agricultural and Food Chemistry, 60*, 9484–9494. https://doi.org/10.1021/jf3015975.

Liu, D., Martínez-Sanz, M., López-Sánchez, P., Gilbert, E. P., & Gidley, M. J. (2017). Adsorption behaviour of polyphenols on cellulose is affected by processing history. *Food Hydrocolloids, 63*, 496–507. https://doi.org/10.1016/j.foodhyd.2016.09.012.

Marlett, J. A., & Vollendorf, N. W. (1994). Dietary fiber content and composition of different forms of fruits. *Food Chemistry, 51*(1), 39–44. https://doi.org/10.1016/0308-8146(94)90045-0.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619–4626. https://doi.org/10.1021/jf010586o.

Padayachee, A., Day, L., Howell, K., & Gidley, M. J. (2017). Complexity and health functionality of plant cell wall fibers from fruits and vegetables. *Critical Reviews in Food Science and Nutrition, 57*(1), 59–81. https://doi.org/10.1080/10408398. 2013.850652.

Padayachee, A., Netzel, G., Netzel, M., Day, L., Mikkelsen, D., & Gidley, M. J. (2013). Lack of release of bound anthocyanins and phenolic acids from carrot plant cell walls and model composites during simulated gastric and small intestinal digestion. *Food & Function, 4*, 906–916. https://doi.org/10.1039/c3fo60091b.

Pérez-Jiménez, J., & Saura-Calixto, F. (2015). Macromolecular antioxidants or non-extractable polyphenols in fruit and vegetables: Intake in four European countries. *Food Research International, 74*, 315–323. https://doi.org/10.1016/j.foodres. 2015.05.007.

Pérez-Jiménez, J., & Saura-Calixto, F. (2018). Fruit peels as sources of non-extractable polyphenols or macromolecular antioxidants: Analysis and nutritional implications. *Food Research International, 111*, 148–152. https://doi.org/10.1016/j.foodres. 2018.05.023.

Phan, A. D. T., Flanagan, B. M., D'Arcy, B. R., & Gidley, M. J. (2017). Binding selectivity of dietary polyphenols to different plant cell wall components: Quantification and mechanism. *Food Chemistry, 233*, 216–227. https://doi.org/10.1016/j.foodchem.2017.04.115.

Pozuelo, M. J., Agis-Torres, A., Hervert-Hernández, D., Elvira López-Oliva, M., Muñoz-Martínez, E., Rotger, R., & Goñi, I. (2012). Grape antioxidant dietary fiber stimulates lactobacillus growth in rat cecum. *Journal of Food Science, 77*(2), H59–H62. https://doi.org/10.1111/j.1750-3841.2011.02520.x.

Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. *Carbohydrate Research, 263*(2), 227–241. https://doi.org/10.1016/0008-6215(94)00175-8.

Renard, C. M. G. C. (2005). Variability in cell wall preparations: Quantification and comparison of common methods. *Carbohydrate Polymers, 60*(4), 515–522. https://doi.org/10.1016/j.carbpol.2005.03.002.

Renard, C. M. G. C., Watrelot, A. A., & Le Bourvellec, C. (2017). Interactions between polyphenols and polysaccharides: Mechanisms and consequences in food processing and digestion. *Trends in Food Science & Technology, 60*, 43–51. https://doi.org/10.1016/J.TIFS.2016.10.022.

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández- Bolaños, J., ... Jiménez, A. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. *Journal of Agricultural and Food Chemistry, 53*, 5212–5217. https://doi.org/10.1021/jf050338i.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science & Technology, 17*(1), 3–15. https://doi.org/10.1016/j.tifs.2005.10.002.

Rodríguez, G., Rodríguez, R., Fernández-Bolaños, J., Guillén, R., & Jiménez, A. (2007). Antioxidant activity of effluents during the purification of hydroxytyrosol and 3,4-dihydroxyphenyl glycol from olive oil waste. *European Food Research and Technology, 224*(6), 733–741. https://doi.org/10.1007/s00217-006-0366-1.

Rubio-Senent, F., Rodríguez-Gutíerrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. *Journal of Agricultural and Food Chemistry, 60*(5), 1175–1186. https://doi.org/10.1021/jf204223w.

Saeman, J. F., Moore, W. E., Mitchell, R. L., & Millett, M. A. (1954). Technique for the determination of pulp constituents by quantitative paper chromatography. *Tappi, 37*, 336–343.

Saura-Calixto, F. (2011). Dietary fiber as a carrier of dietary antioxidants: An essential physiological function. *Journal of Agricultural and Food Chemistry, 59*, 43. https://doi.org/10.1021/jf1036596.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin — A review. *Critical Reviews in Food Science and Nutrition, 37*(1), 47–73. https://doi.org/10.1080/10408399709527767.

Wu, Z., Ming, J., Gao, R., Wang, Y., Liang, Q., Yu, H., & Zhao, G. (2011). Characterization and antioxidant activity of the complex of tea polyphenols and oat β-Glucan. *Journal of Agricultural and Food Chemistry, 59*(19), 10737–10746. https://doi.org/10.1021/jf202722w.

Complex of olive phenolic (hydroxytyrosol and 3,4-dihydroxyphenylglycol) with soluble and insoluble apple dietary fiber with antioxidant activity.

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Abstract.

The aim of this study was to prepare a complex between the olive phenolic compounds, hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG) and their mixture, with soluble and insoluble dietary fiber of apple cell wall. A strong interaction between phenols and apple cell wall occurred with drying and was confirmed by ultraviolet-visible spectrometry, Fourier transform infrared spectrometry, differential scanning calorimetry, thermogravimetry and solid-state ^{13}C-NMR spectroscopy. Although the phenol's antioxidant activity was affected by the cell wall interaction, it was in part restored when the size of the polysaccharide was reduced by enzymatic treatment. *In vitro* assays showed that the complex's antioxidant activity was protected from simulated gastric and intestinal digestion conditions to reach the large intestine, where phenols or fragments of the complex would potentially be released and available for fermentation by gut bacteria. Therefore, HT/DHPG bound to fiber of apple cell wall could be a novel bioactive ingredient in functional food formulations.

Keywords: olive phenols; apple cell wall; antioxidant activity; phenol-soluble dietary fiber complex; phenol-insoluble dietary fiber complex.

1. Introduction.

Interactions between intracellular polyphenols and plant cell walls have recently been described. Specifically, the existence of non-covalent interactions between procyanidins and apple cell wall material was demonstrated and the apple polyphenols were shown to mainly bind to pectin compared to other cell wall components **(Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012**). Also, electrostatic interactions were shown to be important in the interaction between polyphenols and cellulose-based composite and apple cell walls **(Phan, Flanagan, D'Arcy, & Gidley, 2017**). The complexation of polyphenol with polysaccharides plays a key role in regulating the bioaccesibility of polyphenols of fruits and vegetables **(Padayachee, Day, Howell, & Gidley, 2017; Renard, Watrelot, & Le Bourvellec, 2017**). These bound polyphenols reach the colon where they are released and fermented by bacteria into absorbable metabolites that may be essential for maintaining gut health **(Pozuelo et al., 2012; Liu, Martinez-Sanz, Lopez-Sanchez, Gilbert, & Gidley, 2017)**.

Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), are two of the main antioxidant phenols of olive fruit and olive oil with major biological activity. They have been clearly demonstrated to provide remarkable health benefits as they act as antioxidant and have anti-inflammatory, antimicrobial and anticarcinogenic properties (**Fernández-Bolaños, López, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2008; Echevarría, Ortiz, Valenzuela, & Videla, 2017**). These compounds contain an ortho-diphenolic group, with an additional hydroxyl group in the β position in the case of DHPG, which are frequently cited as being important for their radical scavenging and antioxidant properties contributions (**Vilaplana, Auñón, García-Flores, & Gil-Izquierdo, 2014; Garcia-Vilas, Quesada, & Medina, 2017; Zubair et al., 2017**). In previous studies, we demonstrated the potential binding interactions between pectin and HT or DHPG and described the formation of complexes as an efficient system for the delivery of these phenolic antioxidants to the colon **(Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Lama-Muñoz, & Fernández-Bolaños, 2017; Bermúdez-Oria, Rodríguez-Gutiérrez, Rodríguez-Juan, González-Benjumea, & Fernández-Bolaños, 2018;**

Bermúdez-Oria, Rodríguez-Gutiérrez, Fernández-Prior, Vique, & Fernández-Bolaños, 2019). In another recent work we studied the interaction between HT and DHPG with strawberry cell wall, which ocurred during drying and resulted in a strong and irreversible complex. Moreover, the phenols maintained in part their antioxidant activity after binding to the soluble dietary fiber fraction yielded after digestion simulated *in vitro* with gastric and intestinal fluids (**Bermúdez-Oria et al., 2019**).

Based on those findings, the aim of the present study was obtaining better understanding of the mechanisms involved in the formation of such complexes with dietary fiber during drying. Therefore, we investigated the chemical characteristics of HT/DHPG complexed with the soluble and insoluble dietary fiber of apple cell wall by ultraviolet-visible (UV) spectrometry, Fourier transform infrared spectrometry (FT-IR), thermogravimetry analysis (TGA), differential scanning calorimetry (DSC), and solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy. In addition, the *in vitro* antioxidant capacity of such complexes was determined.

2. Material and Methods.

2.1. Isolation of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from olive oil by-products.

HT and DHPG were extracted by hydrothermal treatment at 50–80º C for 60 min from *alperujo,* a by-product of olive oil extraction using the two-phase separation system. After treatment, a liquid phase rich in both compounds was obtained. HT and DHPG were purified by chromatographic fractionation resulting compounds with 90–95% of purity in weight (**Fernández-Bolaños et al., 2013**).

2.2. Preparation of apple cell wall material.

Alcohol-insoluble solids (AIS) from apple fruits were prepared according to the method of **Renard** (2005). Briefly, apples were peeled and cut into 2-3 pieces. The pieces were directly ground in a domestic blender in 70% ethanol and subsequently filtered through a nylon cloth. The resulting solid was ground and washed repeatedly

with 70% ethanol until the filtrate had no color. Drying was performed by solvent exchange (96% ethanol and acetone), then overnight in an oven at 40º C.

2.3. Preparation of the apple cell wall-HT/DHPG complex.

Approximately 500 mg of apple cell wall alcohol-insoluble solid (AIS) was added to 10 mL of 1–10 mg/mL HT or DHPG solutions (corresponding to an initial amount of 200 mg bioactive compound (BC)/g cell wall) or a mixture of 5 mg/mL of HT and 5 mg/mL of DHPG solution (100 mg of HT: 100 mg of DHPH/g cell wall). After overnight swelling, the samples were dried by in the oven for 72 h at 60 ºC. After drying, the cell wall was rehydrated with water and then washed copiously with ethanol 70% to remove the free phenols, and the cell wall/phenol complex separated by filtration. The content of free HT and DHPG was measured by HPLC and the bound phenols calculated by the difference.

2.4. Proximate composition of cell wall.

Non-cellulosic sugar composition was determined by hydrolysis with 2 N trifluoracetic acid (TFA) at 121º C for 1 h. The released sugars were quantified after reduction and acetylation by gas chromatography (GC) **(Englyst & Cummings, 1984**). glucitol by gas chromatography, subtracting the glucose non-cellulosic material. Chromatographic conditions utilized were described by **Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández-Bolaños**, (2012). Uronic acid was quantified using the phenyl-phenol method after sulfuric acid hydrolysis **(Blumenkrantz & Asboe-Hansen, 1973**).

2.5. In vitro gastrointestinal digestion with simulated gastric and intestinal fluids.

HT/DHPG-bound cell wall, free of soluble phenols, was immersed in 100 mL 0.1 M HCl solution at pH 1.2 (simulated gastric fluid) and incubated with gentle shaking in a water bath at 37º C for the first 2 h. After incubation, the sample was filtered with filter paper, and the filtrate used for the quantification of HT and DHPH delivered in gastric

fluid. The insoluble fraction was adjusted to pH 6.8 with the addition of 100 mL phosphate buffer solution (simulated intestinal fluid). The samples were incubated for another 3 h in a water bath at 37º C with agitation. Thereafter, the samples were filtered to separate the soluble fraction from the insoluble fraction, and the free HT and DHPG in the soluble were quantified and the bound phenols calculated by the difference.

2.6. Antioxidant activity measured by DPPH, ABTS and ORAC assays.

2.6.1. Antiradical activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH).

Free radical-scavenging capacity was measured using the DPPH method as described previously (**Rodríguez et al., 2005**). Briefly, Aliquots of 5 µL of each extract, and their dilutions and 195 µL of the DPPH solution (3.8 mg/50 mL of DPPH solution in methanol (stable radical of 2,2-diphenyl-1-picrylhydrazyl) were placed in each microplate well in triplicate. For each sample, a blank with methanol instead of DPPH solution was included. For the determination of the antiradical activity of extracts, a microplate reader (550 model from Bio-Rad, Hercules,CA) was used. After 30 min of reaction, the absorbance at 490 nm was measured.

The results were expressed as the average of the ratios of the slopes of the lines obtained for each sample with Trolox calculated for this wavelengths. The results were expressed in terms of Trolox equivalent antioxidant capacity in µmol Trolox/g of sample.

In the case of insoluble material, the antioxidant activity was evaluated as described by **Fuentes-Alventosa et al.,** (2009) with slight modifications. Briefly, 2–6 mg of insoluble material was suspended in 1 mL of $DPPH^{\bullet}$ reagent (3.8 mg/50 mL methanol). After 30 min of continuous agitation, the material was centrifuged, and the absorbance of the supernatant was measured at 490 nm.

2.6.2. Antiradical activity: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS).

The ABTS assay was performed according to the method of **Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, & Fernández-Bolaños** (2012). This assay is based on the scavenging of ABTS radical ($ABTS^{\cdot+}$) by antioxidants. Briefly, the concentration of ABTS radical was adjusted with ethanol 80–% (v/v) to an initial absorbance of 0.700 ± 0.020 at 734 nm. Aliquots of 13 µL of each phenolic extract, and their dilutions were added to 187 µL of the $ABTS^{\cdot+}$ solution in a 96-well microplate in triplicate. For each sample, a blank with ethanol instead of $ABTS^{\cdot+}$ solution was included. After 30 min of reaction, the absorbance at 400 nm was measured.

The results were expressed in terms of Trolox equivalent antioxidant capacity in µmol Trolox/g of sample.

2.6.3. Antioxidant activity: Oxygen radical absorbance capacity (ORAC) assay.

The ORAC assay is based upon the inhibition of peroxylradical-induced oxidation initiated by the thermal decomposition of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH). The reactive oxygen species (ROS) generated from this thermal decomposition quenches the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples were assayed according to **Ou, Hampsch-Woodill, & Prior** (2001) with minor modifications. Samples were diluted with sodium phosphate buffer (10 mM, pH 7.4) and 25 µL of sample was transferred to a microplate. The blank well received 25 µL phosphate buffer while standards received 25 µL Trolox solutions (10–140 µM). Then 150 µL of 1 µM fluorescein was added to all wells. After incubation (37º C, 15 min), 25 µL AAPH (250 mM) was added to each well to initiate the reaction and a reading taken every 5 min for 90 min (Ex. 485 nm, Em. 538 nm) in a microplate reader (Fluoroskan Ascent™, Thermo Scientific™). Results were calculated using the difference of areas under the fluorescein decay curve between the blank and the sample and expressed as µmol Trolox equivalents/g of sample.

2.7. Enzymatic hydrolysis of HT/DHPG-bound soluble dietary fiber and ultrafiltration.

The hydrolysis of soluble complex was carried out using a mixture of pectinolytic enzymes (4 µg/mL) including endo- and exo-polygalacturonase and pectin esterase (Novo Nordisk, Bagsvaerd, Denmark). The mixture (50 ml) was incubated at 37º C for 24 h and the hydrolysis was terminated by heating to 100º C for 10 min,

The hydrolyzed fraction was subjected to successive ultrafiltration using an Amicon 8400 stirred cell (Millipore Corporation, Bedford, MA, USA) through a molecular weight cut-off of 3000 and 1000 Da. Each retained solution was washed with water until 300 mL of permeate was collected. Three fractions were obtained: the retained fractions > 3000 Da, and > 1000 Da, and the eluted fraction < 1000 Da. All fractions were analyzed for antiradical activity by DPPH, ABTS and ORAC assay.

2.8. Characterization of cell wall-phenol complex.

UV Spectrometry. UV spectroscopy was performed using a Coulter DU 800 UV/visible spectrophotometer (Beckman, USA) in the range 200-400 nm.

FT-IR Spectrometry. FT-IR spectra of samples were obtained using FT-IR Bomem MB-120 spectrophotometer (ABB, Canada) in the range 4000-350 cm^{-1} by the KBr method.

Differential Scanning Calorimetry and themogravimetry analysis. Measurements were carried out with a thermal analyzer (Q20 DSC, TA Instruments, New Castle, DE). The samples were heated from 50 to 275 °C at a heating rate of 10 °C min^{-1} in a nitrogen atmosphere. Also the analysis was preformed using a Q600SDT (TA Instruments, New Castle, DE), with a temperature range between 50 ºC and 500 ºC at a heating rate of 5 ºC min^{-1}, under nitrogen atmosphere. For the themogravimetric analysis a Q600SDT (TA Instruments, New Castle, DE) was used. Approximately 10 mg of sample material was heated from 50 ºC to 500 ºC at a heating rate of 5 ºC min^{-1}, under nitrogen atmosphere.

^{13}C CP/MAS NMR spectroscopy

For the molecular characterization, solid-state ^{13}C nuclear magnetic resonance (NMR) spectra were acquired, using a Bruker Avance III HD 400 MHz spectrometer operating at a ^{13}C frequency of 100.6 kHz and applying the cross polarization magic angle spinning (CP/MAS) technique. Therefore, Zirkonium rotors with KELF-caps with an outer diameter of 4 mm were spun in a triple resonance probe at the magic angle with a spinning speed of 14 kHz. A ramped ^{1}H- pulse was applied during the contact time of 1 ms. Using a pulse delay time of 2 s between 15000 and 81 000 scans were accumulated to achieve adequate signal-to-noise ratios of the spectra.

3. Results and discussion.

3.1. Preparation of the hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) complex with apple cell wall (dietary fiber).

The influence of drying on the formation of a complex of HT/DHPG-bound to the cell wall of strawberries was investigated in a previous study (**Bermúdez-Oria, et al. 2019**). To confirm that such a complex formation occurs also with apple cell walls, we assayed the homogenization of apple cell wall, as an alcohol insoluble solid, in the presence of 10 mg/mL HT and DHPG solutions or a mixture of HT:DHPG of 5 mg/mL each one and further drying. The content of free HT and DHPG was measured by HPLC and the bound phenols calculated by the difference (**Figure 1**). There was a higher retention of HT and DHPG by apple cell wall (52 and 72%) than previously observed for strawberry cell wall (40 and 47%), respectively. In the case of the HT:DHPG mixture, the retention was intermediate between HT and DHPG, at 61.5%. Drying enhanced the binding of apple cell wall with HT/DHPG, as occurred with strawberry cell wall; whereas it is documented that boiling and drying decreases the binding affinity of apple cell walls for procyanidins due to pectin solubilization and degradation, and by altering the cell wall surface area **(Le Bourvellec et al., 2012; Liu et al., 2017)**.

When the HT/DHPG-bound cell wall (dietary fiber) was digested *in vitro* using simulated gastric fluid (pH 1.2, 0.1 M HCl solution, 2 h) with a subsequent pH change,

simulating intestinal fluid (pH 6.8 phosphate buffer, 3 h), the amount of HT and DHPG released was very high, in contrast to the result for strawberry cell wall, from which there was practically no release. In this case, the interactions between BC and apple cell wall seemed much weaker once oven-dried and were released by gastric and intestinal conditions more easily to give a final result of 37% and 47% total retention of HT and DHPG, respectively, coinciding with the retention in the case of strawberry cell wall (**Bermudez-Oria et al.,2019**). The dissolved fraction of phosphate buffer was separated from the insoluble fraction, with both fractions showing a high retention of HT and DHPG, confirmed by the brown color that comes from the initial compounds, which was retained in both fractions (**Figure 1**).

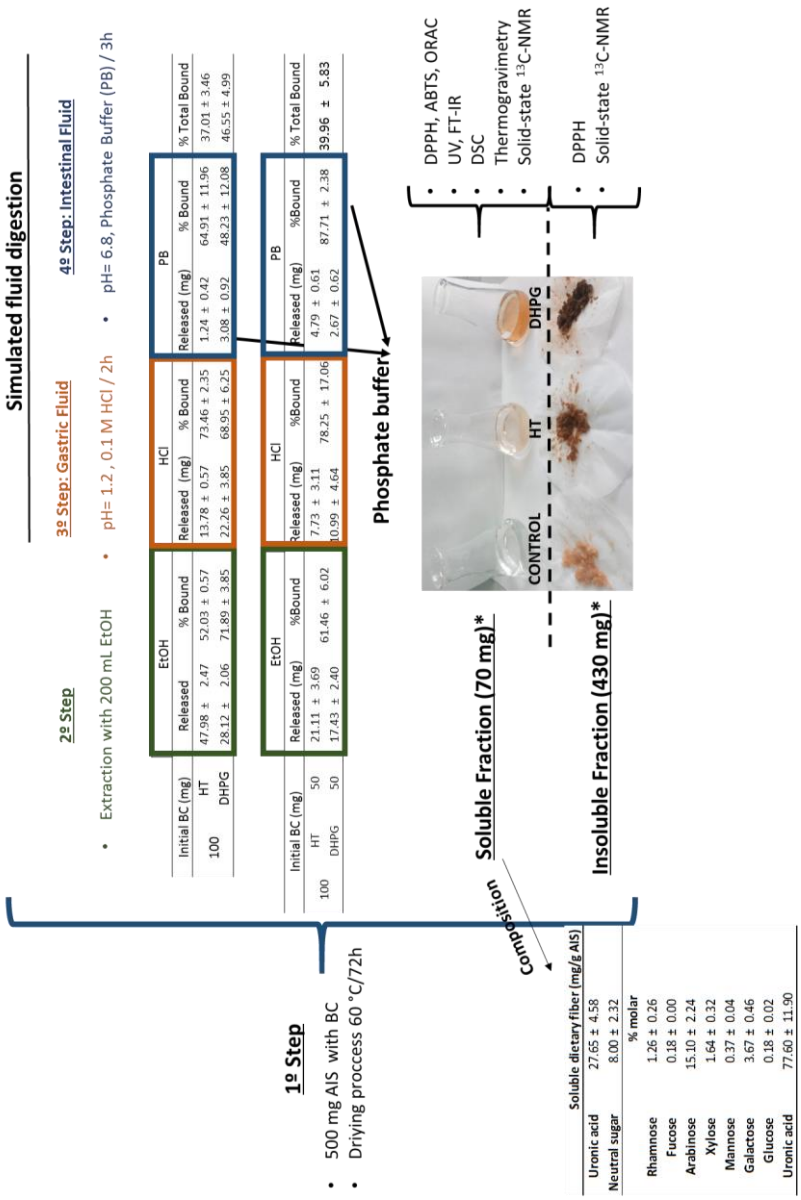


Figure 1. Scheme of the *in vitro* simulated gastrointestinal digestion process of HT/DHPG or their mixture HT:DHPG binding to apple dietary fiber. Percentage of bioactive compounds (BC) bound to AIS during drying process (oven-dried), after release with ethanol, and bound in each step of digestion simulation with respect to the bound BC. *Represents the average weight of the insoluble and soluble fractions obtained from digestion of HT/DHPG-bound cell wall (n = 3 for HT and n = 3 for DHPG). The table lists the composition (mg/g AIS) of the HT/DHPG-soluble dietary fiber complex. Each value is the

Based on monosaccharide analysis of the soluble fraction and its uronic acid content (**Figure 1**), the predominant component of the HT/DHPG-bound complex was a pectin rich in arabinose. This is in agreement with our previous reports of strong binding between HT and DHPG with pectinate beads **(Bermúdez-Oria et al., 2017; Bermúdez-Oria et al., 2018**) or with the soluble dietary fiber of strawberry (**Bemúdez-Oria, et al., 2019**). Therefore, soluble fibers, such as pectin, with associated antioxidant compounds, could be of interest to the food industry due to their health benefits **(Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006**) and potential technological applications **(Thakur, Singh, Handa, & Rao, 1997**).

3.2. Physical and chemical characterization of the HT/DHPG complex with soluble and insoluble dietary fiber.

3.2.1. UV Absorption Spectrum.

In the absorption spectra of the complex of HT and DHPG in the soluble fraction (**Figure 2a**), a slight band was found at 280 nm (π-π* transition of the phenolic group), and was slightly more pronounced in the case of DHPG, but not present in the control spectrum (soluble fraction obtained from apple cell wall with no addition of BC).

3.2.2. FT-IR Analysis.

The FT-IR spectra of the soluble fraction confirmed the characteristic absorption bands of pectin: at 3000-3800 cm^{-1} and 2900 cm^{-1}, which are attributed to OH- and C-H stretching vibrations, respectively (**Figure 2b**). The peaks at 1100, 1700, and 1745-1760 cm^{-1} are assigned to –C-O-C-, C=O stretching, and ester carbonyl stretching vibrations, respectively. No significant differences were observed in the corresponding spectra of the complex of HT/DHPG with the soluble fraction compared with the control FT-IR spectrum. A total disappearance of the characteristic bands of phenolic compounds were observed in the complex spectra, which may be attributed to the complexation and, partially, to the low loading content of BC in relation with the pectin macromolecule content. Only a slight difference in the zone of 600-670 cm^{-1}, associated with C-H bonds of flexion out-of plane of the aromatic compounds, was observed. This

is a reliable indicator of the incorporation of HT/DHPG into the complex, although this result does indicate the type of involved binding.

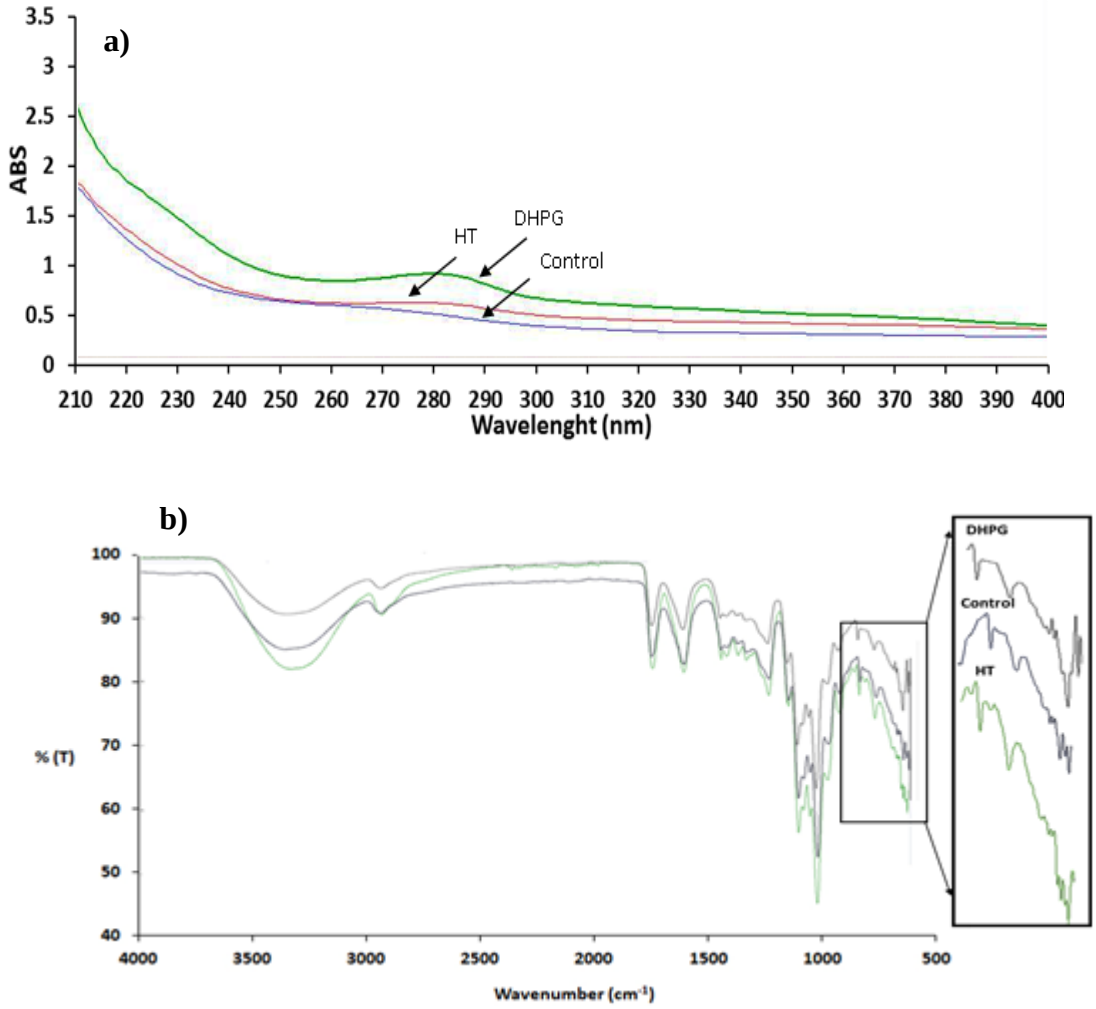
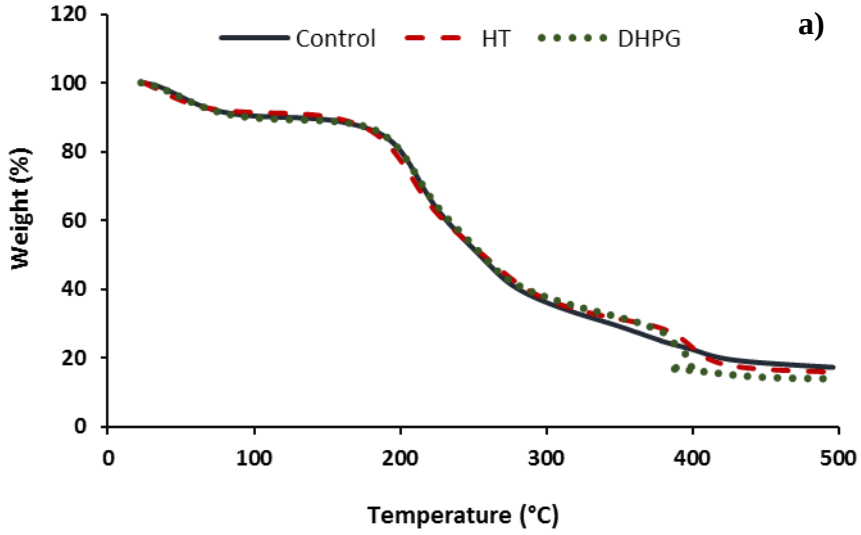


Figure 2. Ultraviolet-visible (UV) (a) and Fourier transform infrared (FT-IR) (b) spectra of HT/DHPG–soluble dietary fiber complex and comparison with control (soluble dietary fiber with no BC).

3.2.3. Thermogravimetric (TGA) and differential scanning calorimetry (DSC) analysis.

TGA analysis was performed on the control and the two complexes containing BC samples. TGA plots showed three regions at 25-100º C, 150-350º C and 350-500º C

(**Figure 3a**), as reported by other authors for pectin (**Combo et al., 2013**). The first region (25-100º C) was attributed to the water loss during the temperature rise, and the second region (150-350º C) corresponded to a rapid mass loss due to the polysaccharide decomposition, with complete decomposition of the pectin at 240º C. The third region (350-500º C), the only region that showed differences between the control and HT/DHPG complex samples, showed a slow mass loss after volatilization and thermal decomposition of other components with the subsequent formation of solid char and various gaseous products. The decomposition of the BC linked to the soluble fraction was observed above 400º C.



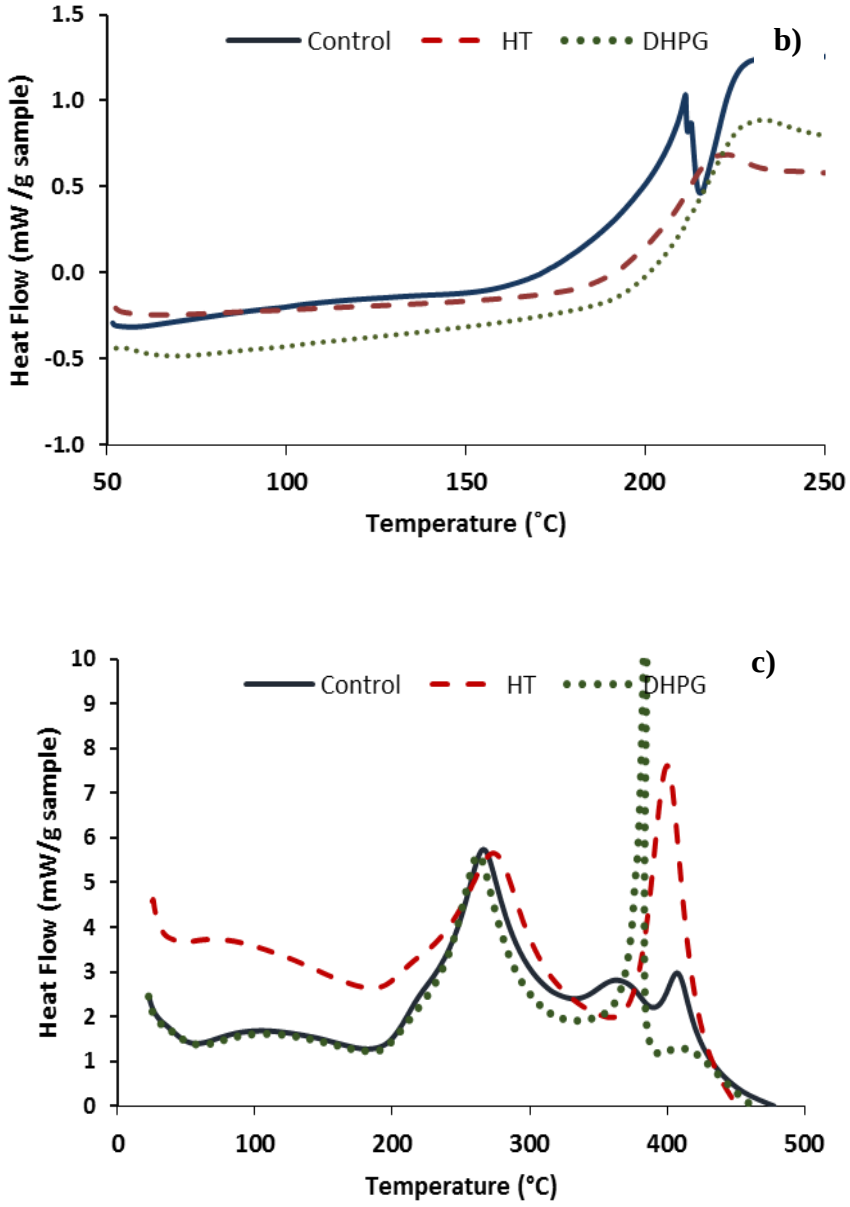


Figure 3. Thermogravimetry analysis (TGA) (a) and differential scanning calorimetry (DSC) (b and c) thermograms of HT/DHPG–soluble dietary fiber complex and comparison with control (soluble dietary fiber with no BC).

The control's DSC thermogram showed an endothermic peak around 215º C that corresponded to the melting temperature and was not observed in the DSC thermogram of HT and DHPG complexes in the scanned range in the assay conditions (**Figure 3b**). Furthermore, the DSC plot revealed a slight exothermic reaction in the control and HT/DHPG samples, corresponding to the degradation of the polysaccharide, which began around 230º C (**Figure 3c**), with a maximum at 260º C and a slight shift at a higher temperature observed for the HT-bound complex. Besides, the intensity of flow of heat observed in the HT and DHPG soluble complexes were several times higher than for the control, revealing significant changes due to the presence of BC at these high temperatures.

3.2.4. Solid-state ^{13}C NMR spectroscopy.

The solid-state ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance CP/MAS NMR technique was used to investigate interactions between the added phenols, HT and DHPG, and the soluble and insoluble dietary fiber of apple. For the dry soluble dietary fraction (**Figure 4**), the resonance lines between 60 and 100 ppm are caused by C1-C5 of the pectin backbone (**Synytsya, Copikova, & Brus, 2003; Marcon, Carneiro, Wosiacki, Beleski-Carneiro, & Petkowicz, 2005; Ng, Zujovic, Smith, Johnston, Schoroder, & Melton, 2014**). The peaks at 174 ppm and 170 ppm can be attributed to esterified and non-esterified carboxyl C (C6) of galacturonic acid esterified and not esterified respectively. The signal at 53 ppm corresponds to metoxyl C at C-6 (**Figure 4**) (**Synytsya et al., 2003; Ng et al., 2014**). Additional peaks detected between 110 ppm and 160 ppm and between 31 and 37 ppm in the NMR spectra of freezed-dried complexes of HT/DHPG-soluble dietary fiber can be assigned to aromatic C. The signals between 130 and 145 ppm, typical for phenol C, suggests that phenols bind to the soluble dietary fiber. Since no major chemical shifts of the pectin signals due to the addition of HT/DHPG are observed, it may be concluded that the complexation caused no major conformational changes.

The ^{13}C CP/MAS NMR spectra of the complex of HT and DHPG with the insoluble fraction did not show significant differences compared to the spectrum of the insoluble

fraction of the control (**Figure 4**), a result, which coincided with the finding of **Phan et al**. (2017) for apples cell walls. In our case, the spectrum is dominated by signals from cellulose, and other neutral polysaccharides, as well as to pectic polysaccharides (Table 1). However, the low intensity is observed in the chemical shift region assignable to carboxyl C, indicates minor contributions of galacturonic acid, if compared to the soluble dietary fiber. Although the spectrum of the insoluble fraction shows some weak intensity in the chemical shift region of phenol C, new resonance lines and a slight increase of signal intensity within this region are observed for the spectra of the complex insoluble material, allowing the conclusion that complexed HT and DHPG are present (**Figure 4**).

Soluble fiber			Insoluble fiber		
Sample		**Chemical shift (ppm)**	**Sample**		**Chemical shift (ppm)**
Pectin	C6 ($COOCH_3$)	174.8	**Pectin**	C6 (COO^-)	170.6
	C6 (COO^-)	170.5	**Cellulose**	C1	104.4
	C1	100.4	**Pectin**	C4	83
	C4	79.3	**Pectin**	C2.3.5	71.5
	C2.3.5	68.0	**Cellulose**	C6	61.7
	O-Me	52.9	**Pectin**	O-Me	52.9
HT/DHPG	C-aromatic	38.4	**HT/DHPG**	C-aromatic	38.4
		144.8			144.8
		143.2			143.2

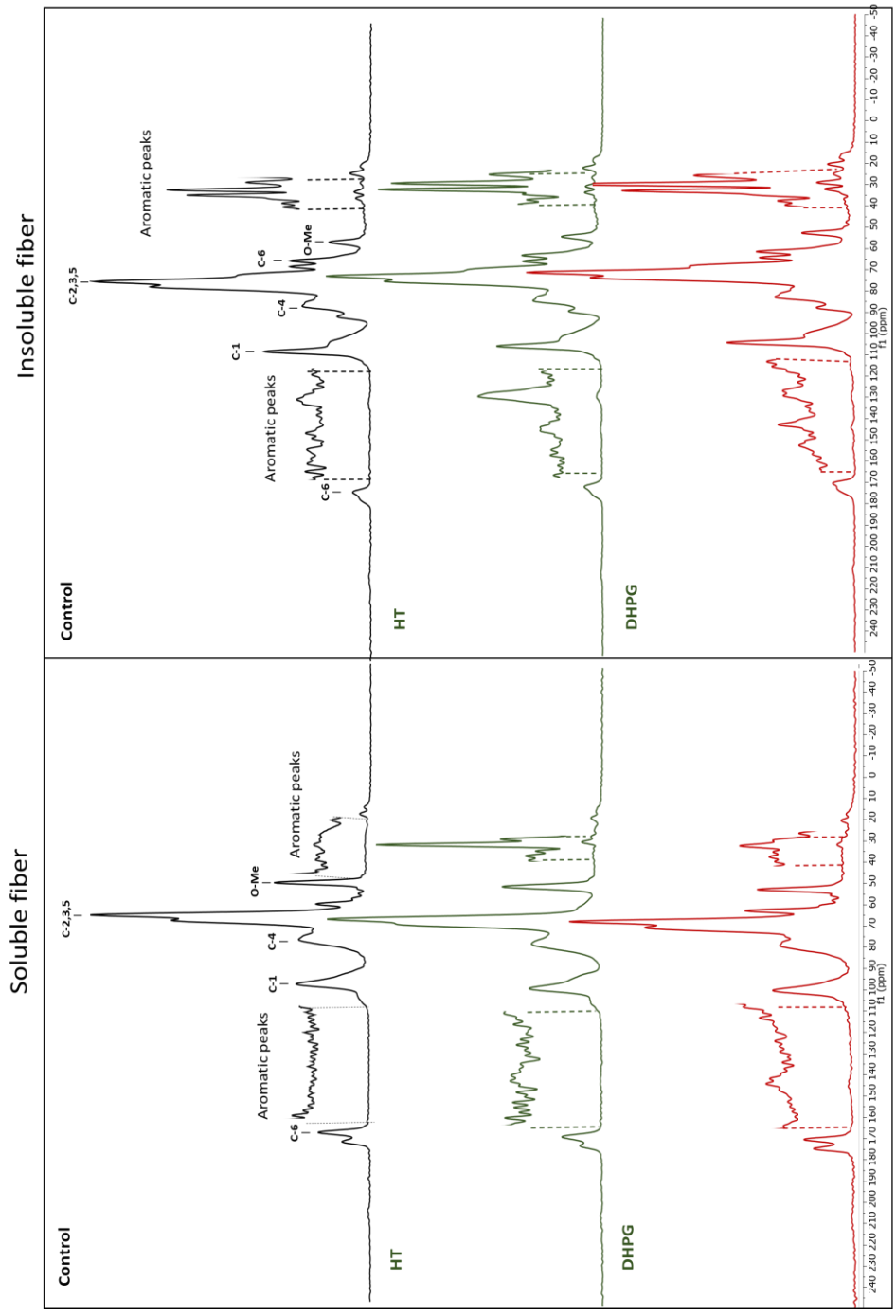


Figure 4. Solid-state ^{13}C NMR spectra of HT/DHPG–soluble and insoluble dietary fiber complex and comparison with a soluble and insoluble dietary fiber with no BC added (Control). Peak assignments in based on the previously reported data.

3.3. Antioxidant activity of the insoluble and soluble fractions in vitro.

The insoluble apple fiber fraction showed very little radical scavenging activity in the assay conditions (**Figure 5**), whereas the fiber complexed with BC (200 mg BC/g cell wall) showed certain antioxidant activity. This activity was highest for linked-DHPG, followed by the mixture HT:DHPG (100:100 mg/g cell wall), and lowest for HT, which needed more fiber (up to 15 mg of insoluble fraction) to obtain an appreciable decoloration of the DPPH free radical. These results with apple cell wall are very different from those obtained in our previous work with insoluble strawberry fiber, in which there was no difference between the samples with HT or DHPG added and the control, although the control did present certain antioxidant activity (**Bermúdez-Oria et al., 2019**). We conclude that negligent amounts of polyphenols were associated to the insoluble dietary fiber of apple, although the addition of the potent phenolic antioxidants HT and DHPG or their mixture led to the formation of a strong complex with the insoluble material of apple cell wall.

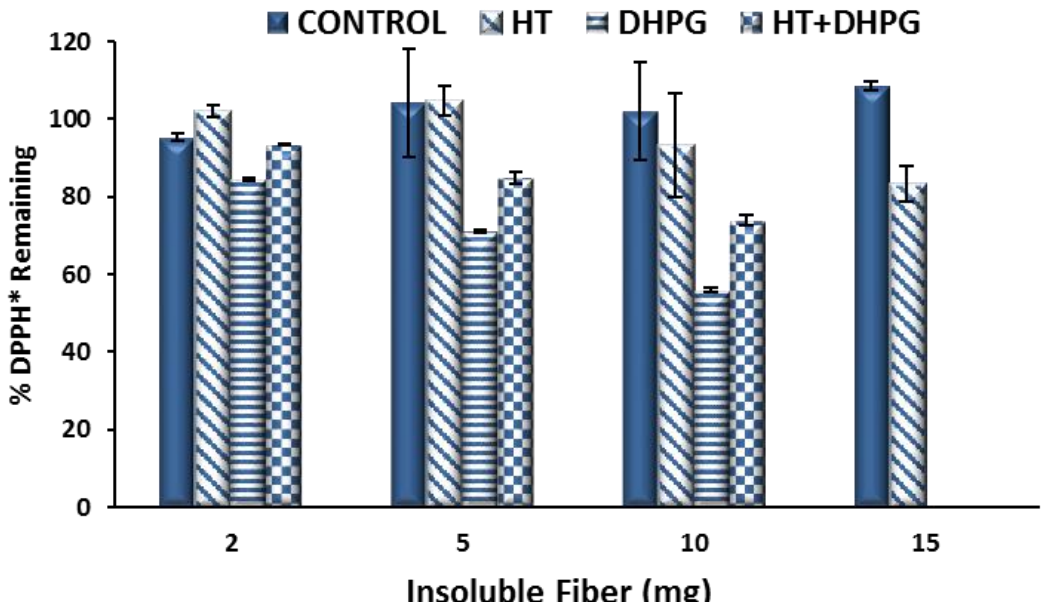


Figure 5. DPPH scavenging activity of insoluble dietary fiber bound to HT (2, 5, 10 and 15 mg) and DHPG or the mixture HT:DHPG (2, 5 and 10 mg), expressed as percent DPPH remaining. Comparison with control (insoluble dietary fiber with no BC). Each bar represents the average value of three replicates. Errors bars represent standard deviation (n = 3).

The antioxidant activity of the *soluble cell wall* fraction was studied by three different methods (DPPH, ABTS and ORAC assays) with the addition of 200 mg of HT and DHPG, and their mixture (100:100 mg of HT:DHPG) added to 1.0 g cell wall (**Figure 6a,b**). In the case of HT-bound soluble dietary fiber or the soluble dietary fiber control, no activity was found using the ABTS and DPPH assays, although activity was observed using the ORAC assay, with significant differences between HT and the control. In contrast, for the DHPG-bound soluble fraction, or the HT:DHPG mixture, free radical scavenging activity was observed by all three methods. These results are in agreement with previous reports on DHPG-pectin complex formation via encapsulation (**Bermúdez-Oria et al., 2018**) and DHPG-bound soluble dietary fiber complex from strawberry cell wall (**Bermúdez-Oria et al., 2019**), which confirmed that the DHPG-linked complexes maintain more antioxidant activity than HT-linked ones. The additional –OH group of DHPG with respect to HT may allow for greater availability of the catechol group, which is responsible for the BCs' antioxidant activity (**Spizirri et al., 2009**). Curiously, the soluble fraction sample with the mixture of HT and DHPG (100:100 mg/g cell wall) showed an important antiradical activity (122 and 403 µmol of Trolox/g sample for DPPH and ORAC assays, respectively), whereas the sum of activity of HT and DHPG for DPPH and ORAC were only 47 and 357 µmol of Trolox/g sample, respectively, using double the amount of BC for the formation of the complex. These results suggest a possible synergistic effect between HT and DHPG.

Therefore, the two potent phenolic antioxidants HT and DHPG form a strong complex with the insoluble and soluble polysaccharides of apple cell wall and impart their antioxidant properties to the complex.

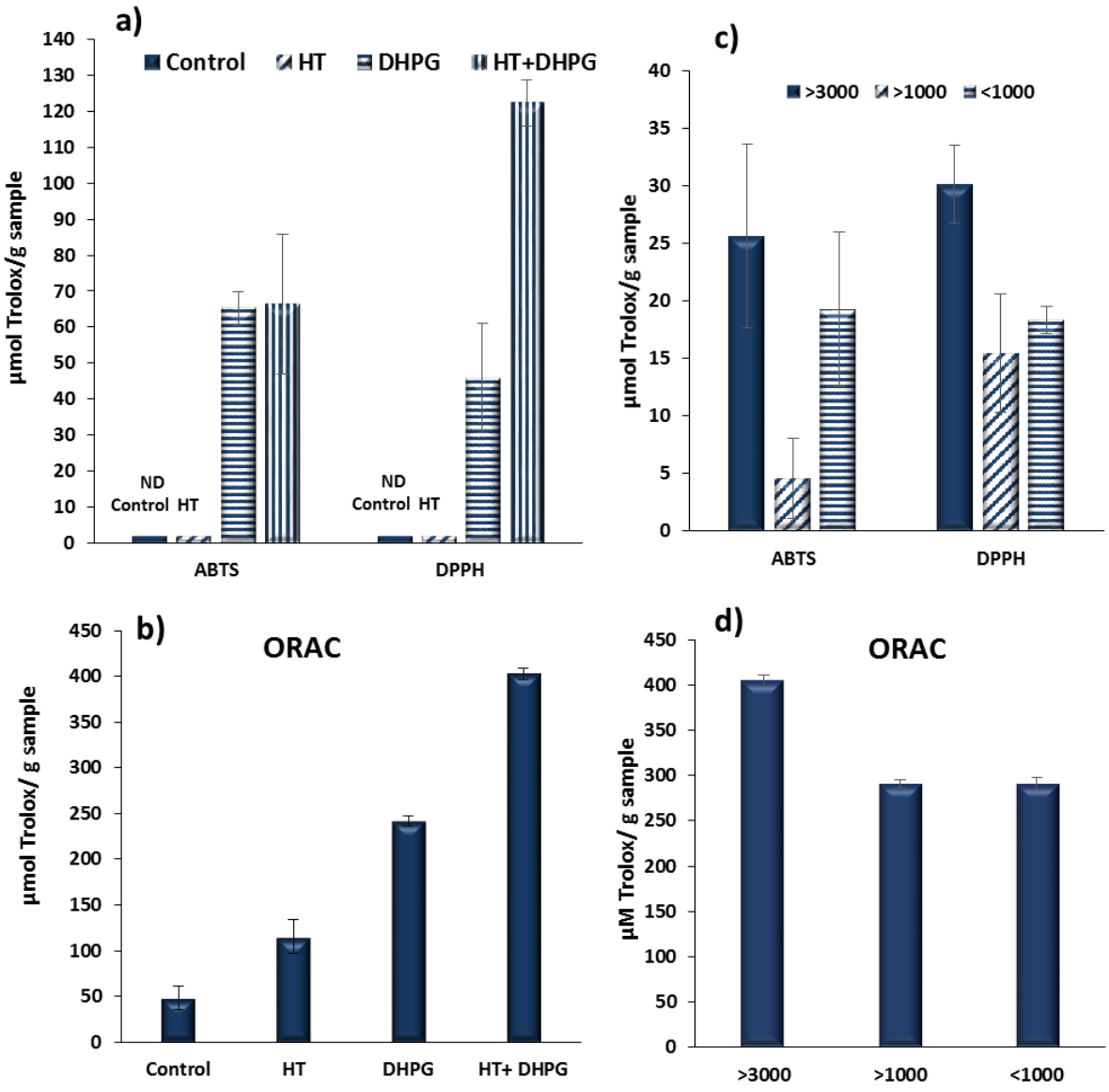


Figure 6. ABTS and DPPH scavening activity (a) and oxygen radical capacity (ORAC)(b) of the HT/DHPG- and their mixture (HT:DHPG)-soluble dietary fiber complex. The three assays are expressed as µmol Trolox/g sample. Antiradical capacity measured by ABTS and DPPH (c) and by ORAC (d) of the pectin fragments obtained from HT:DHPG-soluble dietary fiber complex treated with a mixture of pectinolytic enzymes and recovered by a sequential ultra-filtration through 3000 and 1000 Da molecular weight cut off-membranes. Errors bars represent standard deviation (n = 3).

3.4. Release of pectin fragments with antioxidant activity from the HT:DHPG-soluble dietary fiber complex.

In a previous study, when the size of the polysaccharides in the complex with strawberry cell wall was reduced, the antiradical activity was restored (**Bermúdez-Oria et al., 2019**). Therefore, a mixture of pectinolytic enzymes was added to reduce the molecular size of the HT:DHPG mixture-soluble dietary fiber complex to investigate its effect on the complex's antiradical activity. After enzymatic digestion the solubilized fraction was subjected to sequential ultra-filtration through a 3000 and 1000 Da molecular weight cut-off membrane and the corresponding eluted and retained fractions were analyzed for antiradical activity by DPPH, ABTS and ORAC assays (**Figure 6c,d**). For the ORAC assay, the antioxidant activity of the fraction > 3000 Da (405 µmol of Trolox/g sample) was similar to the activity of the initial fraction of the HT:DHPG mixture linked to soluble dietary fiber (**Figure 6d**). In contrast, the antioxidant activity was high in the fractions of smaller molecular size, with values of 290 and 291 µmol of Trolox/g for the < 1000 Da and 3000-1000 Da fractions, respectively. The increase of antioxidant activity in complexes of a smaller molecular size indicates that although the antiradical activities of HT and DHPG seem to be directly or indirectly affected by their interaction with polysaccharides, mostly pectin, their antiradical activity is partially restored when the size of the polysaccharides is reduced.

The reduction of the size of the oligomers or the hydrolytic process of soluble dietary fiber or pectin by colonic bacterial enzymes could change the outcomes obtained in this study; however, this result suggests that the hydrolytic process releases oligomers with potential antioxidant activity from this complex. This activity could help to prevent certain kinds of degenerative or chronic diseases such as colon cancer or inflammatory bowel disease (IBD) (**Saura-Calixto, 2011)**, although future research is needed to verify this hypothesis.

4. Conclusions.

This study confirmed the formation of phenol-polysaccharide complexes via drying. We provided experimental evidence that the complexation of HT/DHPH with soluble and insoluble apple cell wall retained antioxidant activity in vitro after simulated gastrointestinal fluid and may be protected from absorption during gastrointestinal transit to reach the colon. Enzymatic treatment and reduction of the size of the polysaccharides in the soluble dietary fiber complex, as a simulation of hydrolysis by colonic microflora, released oligomers with antioxidant activity and partially restored the activity of HT/DHPG affected by the interaction with components of cell wall. As such, the complexes formed between HT and DHPG, which are two natural phenols present in olive fruit, with the cell wall of apple fruits could be optimized as a novel bioactive ingredient in functional food formulations to promote intestinal health.

Declarations of Interest: None.

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5. References.

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Fernández-Prior, A., Vioque, B., & Fernández-Bolaños, J. (2019). Strawberry dietary fiber functionalized with phenolic antioxidants from olives. Interactions between polysaccharides and phenolic compounds. *Food Chemistry, 280*, 310-320. https://doi.org/10.1016/j.foodchem.2018.12.057

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rodríguez-Juan, E., González-Benjumea, A., & Fernández-Bolaños, J. (2018). Molecular interactions between 3,4-

dihydroxyphenylglycol and pectin and antioxidant capacity of this complex in vitro. *Carbohydrate Polymers*, *197*, 260–268. https://doi.org/10.1016/j.carbpol.2018.05.089

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Lama-Muñoz, A., & Fernández-Bolaños, J. (2017). Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting. *Carbohydrate Polymers*, *163,*292–300. https://doi.org/10.1016/j.carbpol.2017.01.027

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New Method for Quantitative Determination of Uronic Acids. *Analytical Biochemistry, 54*(2), 484–489.

Combo, A.M.M., Aguedoa, M., Quiévy, N., Danthinec, S., Goffina, D., Jacquet, N., Blecker, C., Devaux, J., & Paquot, M. (2013). Characterization of sugar beet pectic-derived oligosaccharides obtained by enzymatic hydrolysis. *International Journal of Biological Macromolecules, 52*, 148-156.

Echeverría, F., Ortiz, M., Valenzuela, R., & Videla L. A. (2017). Hydroxytyrosol and cytoprotection: A projection for clinical interventions. International Journal of Molecular Sciences, 180, 930.

Englyst, H. N., & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *The Analyst*, *109*(7), 937. https://doi.org/10.1039/an9840900937

Fernández-Bolaños, J. G., López, Ó., Fernández-Bolaños, J., & Rodríguez-Gutiérrez, G. (2008). Hydroxytyrosol and derivatives: Isolation, synthesis, and biological properties. *Current Organic Chemistry*, *12*(6), 442–463. https://doi.org/10.2174/138527208784083888

Fernández-Bolaños, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J. M., Maya-Castilla, I., Rubio-Senent, F., Marset Castro, A. (2013). Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroxyphenylglycol extract, and hydroxytyrosyl acetate extract from by-products

of the olive tree and the purification of thereof. International Patent No. WO 2013/007850A1.

Fuentes-Alventosa, J. M., Rodríguez-Gutiérrez, G., Jaramillo-Carmona, S., Espejo-Calvo, J. A., Rodríguez-Arcos, R., Fernández-Bolaños, J., … Jiménez-Araujo, A. (2009). Effect of extraction method on chemical composition and functional characteristics of high dietary fibre powders obtained from asparagus by-products. *Food Chemistry*, *113*(2), 665–671. https://doi.org/10.1016/j.foodchem.2008.07.075

García-Vilas, J. A., Quesada, A. R., & Medina, M. A. (2017). Hydroxytyrosol targets extracellular matrix remodeling by endothelial cells and inhibits both ex vivo and in vivo angiogenesis. Food Chemistry, 221, 1741-1746.

Graciani, E., & Vázquez, A. (1980). Estudio de los componentes del aceite de oliva por cromatografía líquida de alta eficacia (HPLC). II Cromatografía en fase inversa. *Grasas y Aceites*, *31*, 237–243.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids*, *28*(1), 92–104. https://doi.org/10.1016/J.FOODHYD.2011.11.008

Le Bourvellec, C., Watrelot, A. A., Ginies, C., Imberty, A., & Renard, C. M. G. C. (2012). Impact of Processing on the Noncovalent Interactions between Procyanidin and Apple Cell Wall. *Journal of Agricultural and Food Chemistry, 60*, 9484–9494. https://doi.org/10.1021/jf3015975

Liu, D., Martinez-Sanz, M., Lopez-Sanchez, P., Gilbert, E. P., & Gidley, M. J. (2017). Adsorption behaviour of polyphenols on cellulose is affected by processing history. *Food Hydrocolloids*, *63*, 496–507. https://doi.org/10.1016/J.FOODHYD.2016.09.012

Marcon, M. V., Carneiro, P. I. B., Wosiacki, G., Beleski-Carneiro, E., & Petkowicz, C. L. O. (2005). Pectins from apple pomace– characterization by 13C and 1H NMR

spectroscopy. *Annals of Magnetic Resonance, 4*, 56-63.

Ng, J.K.T., Zujovic. Z.D., Smith, B.G., Johnston, J.W., Schröder, R., & Meltona, L.D. (2014). Solid-state 13C NMR study of the mobility of polysaccharides in the cell walls of two apple cultivars of different firmness. *Carbohydrate Research, 386*, 1-6.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry, 4*9(10), 4619–4626. https://doi.org/10.1021/jf010586o

Padayachee, A., Day, L., Howell, K., & Gidley, M. J. (2017). Complexity and health functionality of plant cell wall fibers from fruits and vegetables. *Critical Reviews in Food Science and Nutrition*, *57*(1), 59–81. https://doi.org/10.1080/10408398.2013.850652

Phan, A. D. T., Flanagan, B. M., D'Arcy, B. R., & Gidley, M. J. (2017). Binding selectivity of dietary polyphenols to different plant cell wall components: Quantification and mechanism. *Food Chemistry*, *233*, 216–227. https://doi.org/10.1016/j.foodchem.2017.04.115

Pozuelo, M. J., Agis-Torres, A., Hervert-Hernández, D., Elvira López-Oliva, M., Muñoz-Martínez, E., Rotger, R., & Goñi, I. (2012). Grape Antioxidant Dietary Fiber Stimulates Lactobacillus Growth in Rat Cecum. *Journal of Food Science*, *77*(2), H59–H62. https://doi.org/10.1111/j.1750-3841.2011.02520.x

Renard, C. M. G. C. (2005). Variability in cell wall preparations: quantification and comparison of common methods. *Carbohydrate Polymers, 60*(4), 515–522. https://doi.org/10.1016/j.carbpol.2005.03.002

Renard, C. M. G. C., Watrelot, A. A., & Le Bourvellec, C. (2017). Interactions between polyphenols and polysaccharides: Mechanisms and consequences in food processing and digestion. *Trends in Food Science & Technology*, *60*, 43–51. https://doi.org/10.1016/J.TIFS.2016.10.022

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández- Bolaños, J., & Jiménez, A. (2005). Antioxidant activity of ethanolic extracts from several

asparagus cultivars. *Journal of Agricultural and Food Chemistry, 53,* 5212−5217. https://doi.org/10.1021/jf050338i

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science & Technology, 17*(1), 3–15. https://doi.org/10.1016/j.tifs.2005.10.002

Rubio-Senent, F., Rodríguez-Gutíerrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New Phenolic Compounds Hydrothermally Extracted from the Olive Oil Byproduct Alperujo and Their Antioxidative Activities. *Journal of Agricultural and Food Chemistry*, *60*(5), 1175–1186. https://doi.org/10.1021/jf204223w

Saura-Calixto, F. (2011). Dietary Fiber as a Carrier of Dietary Antioxidants: An Essential Physiological Function. *Journal of Agricultural and Food Chemistry*, *59*, 43. https://doi.org/10.1021/jf1036596

Saeman, J. F., Moore, W. E., Mitchell, R. L., & Millett, M. A. (1954). Technique for the determination of pulp constituents by quantitative paper chromatography. *Tappi, 37*, 336–343.

Spizzirri, U. G., Iemma, F., Puoci, F., Cirillo, G., Curcio, M., Paris, O. I., & Picci, N. (2009). Synthesis of antioxidant polymers by grafting of gallic acid and catechin on gelatin. *Biomacromolecules, 10(7),* 1923–1930.

Synytsya, A., Copíková, J., & Brus, J. (2003). 13C CP/MAS NMR spectra of pectins: a peak-fitting analysis in the C-6 region. *Czech Journal of Food Science*, *21*, 1-12.

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, M. A. (1997). Chemistry and uses of pectin — A review. *Critical Reviews in Food Science and Nutrition*, *37*(1), 47–73. https://doi.org/10.1080/10408399709527767

Vilaplana-Pérez, C., Auñón, D., García-Flores, L. A., & Gil-Izquierdo, A. (2014). Hydroxytyrosol and Potential Uses in Cardiovascular Diseases, Cancer, and AIDS. *Frontiers in Nutrition, 1,* 18.

Zubair, H., Bhardwaj, A., Ahmad, A., Srivastava, S. K., Khan, M. A., Patel, G. K., Singh, S., & Singh, A. P. (2017). Hydroxytyrosol induces apoptosis and cell cycle arrest

and suppresses multiple oncogenic signaling pathways in prostate cancer cells. *Nutrition and Cancer, 69*, 932–942.

BLOQUE IV

Título: Olive extract rich in polyphenols and polysaccharides with antioxidant and antiproliferative activity on bladder cancer cells

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Publicación: Journal and Funtional Foods (Bajo revision)

Título: Pectin-rich extracts from olives inhibit proliferation of Caco-2 and THP-1 cells.

Autores: Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, Manuel Alaiz, Javier Vioque, Julio Girón-Calle & J Juan Fernández-Bolaños

Publicación

Título: Polyphenols associated to pectic polyssacharides account for most of the antiproliferative and antioxidant activities in olive extracts..

Autores Alejandra Bermúdez-Oria, Guillermo Rodríguez-Gutiérrez, Manuel Alaiz, Javier Vioque, Julio Girón-Calle & Juan Fernández-Bolaños

Publicación

Resumen Bloque IV

Las pectinas representan una familia de complejos heteropolisacáridos aniónicos presentes en las paredes celulares de vegetales, frutas y otras plantas. La principal fuente de obtención de las pectinas son frutas cítricas (CP) o manzana, aunque actualmente se estan introudciendo otras fuentes a partir de los residuos de las indrustrias agroalimentarias, como puede ser el caso del alperujo. Debido a que la pectina es una fibra soluble, ésta no es digerida por el ser humano, sin embargo las llamadas pectinas modificadas (MCP) sí lo son. MCP son pectinas de peso molecular más bajo obtenidas por tratamiento enzimático, químico o térmico de pectina cítrica (CP) lo cual produciría pectinas de peso molecular de alrededor de 10 kDa, las cuales teóricamente sí podrían absorberse y por tanto circular por el torrente sanguíneo. Estudios indican que gracias a los azúcares neutros que la pectina presenta en su estructura, ésta se puede unir y bloquear a una proteína prometastásica y antiapopotótica llamada Galectina-3

Actualmente, la mayoría de las investigaciones realizadas con PM emplean pectinas obtenidas a partir de cítricos (MCP). Las pectinas utilizadas en nuestros estudios fueron obtenidas a partir del alperujo mediante tratamiento con vapor directo y con diferentes post-tratamientos. Fueron aislados y caracterizados extractos de a polisacáridos de bajo peso molecular ricos en pectinas con fenoles asociados, obtenido del alperujo.

Estos extractos exhibieron una fuerte actividad antioxidante ORAC [1,3*], así como un importante efecto sobre la proliferación de células de carcinoma de colon (Caco-2)[2,3*] y líneas celulares de leucemia monocítica (THP-1)[2,3*]. Además de presentar activación de caspasa en líneas THP-1[2*]. Mientras que se estudió también el efecto sobre cuatro líneas de cáncer vesical (RT112, T24 J82 y SCaBER)[1*]. Además, para el caso de estas líneas de cáncer vesical, los efectos antiproliferativos de los extractos obtenidos del alperujo fueron comparados con un conocido agente quimioterapéutico como el cisplatino (CDDP)[1*]. Los resultados obtenidos

mostraron una importante reducción de la proliferación celular a niveles iguales o superior que el CDDP, para SCaBER y T-24, y niveles muy inferiores al de MCP para todas las líneas celulares. Así mismo, si se adicionó de forma secuencial, extractos ricos en pectinas del alperujo con el CDDP, y se observó como el efecto citotóxico del cisplatino aumenta sobre estas líneas de cáncer vesical, lo que sugiere el uso potencial de las pectinas modificadas como un agente adjunto para la quimioterapia con cisplatino. Además, nuestros resultados confirman que los extractos obtenidos del alperujo inhiben significativamente la hemaglutinación y podrían desarrollarse como un nuevo inhibidor potencial de galectina-3, además este estudio reveló el posible papel importante en el efecto antiproliferativo de Galectina-1.

Diferentes estudios indican que dependiendo del método de extracción las propiedades de las pectinas serán diferentes. Por tanto, se obtuvieron extractos ricos en pectinas y fenoles a diferentes tiempos y temperaturas mediante tratamiento térmico con vapor directo. Con objeto de clarificar a que era debido la elevada actividad antioxidante y antiproliferativa encontrada, se llevó a cabo un blanqueamiento de los extractos con clorito sódico para eliminar los compuestos fenólicos. Observándose, cómo la actividad antioxidante y antiproliferativa se redujo considerablemente en los extractos blanqueados, los cuales se comportaban de forma similar a la actividad mostrada por la MCP tanto en células de carcinoma de colon (Caco-2) como en líneas celulares de leucemia monocítica (THP-1).

Esquema resumen bloque IV.

Olive extract rich in polyphenols and polysaccharides with antioxidant and antiproliferative activity on bladder cancer cells.

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ABSTRACT

The objective of this study was the extraction and characterization of newly extracted pectin from “alperujo”, the by-product of the extraction of olive oil, and evaluate the impact of this olive extracts on bladder cancer alone or combined with cisplatin The extracts, named Pectoliv, with high polyphenol content associated to polysaccharides rich in pectin, exhibited strong antioxidant activity in the oxygen radical absorbance capacity (ORAC) assay as well as an important anti-proliferative capacity in vitro against four human bladder cancer cells lines, RT112, T24, J82, and SCaBER. We also demonstrated that Pectoliv treatment reduces the expression of galectin-1 and galectin-3, especially when these galectin-1 and -3 expressions was significantly increased with cisplatin. Moreover, Pectoliv enhanced the cytotoxic effect of cisplatin, suggesting its potential usefulness as an adjunct agent to cisplatin and significantly inhibited the agglutination of erythrocytes and thus may have the potential for development as a novel galectin-3 inhibitor.

Keywords: bladder cancer; modified pectin; alperujo; proliferation; cisplatin; galectin-3

1. Introduction

The olive oil manufacturing process, by the continuous two-phase extraction system, generates oil and a by-product that is a combination of liquid (olive vegetative water) and solid (skin, peel, pulp and piece of stones) olive-pomace waste, called "alperujo". The annual production of alperujo in Spain alone reaches 4–6 million tons. Alperujo is a promising source of substances with high added value, including phenolic compounds, which represent 98% of the total phenols in the olive fruit, while the remaining 2% confer the bioactive properties of olive oil (**Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2013**). Another important component of olive pomace is the cell wall material that is mainly composed of cellulose, xylan, glucurunoxylan, xyloglucan and pectic material rich in arabinose (**Jiménez, Guillén, Fernández-Bolaños, & Heredia, 1994**)(**Coimbra, Cardoso, & Lopes-da-Silva, 2010**). The crushing of olives forms the olive paste, and its slow mixing at moderate temperature (milling and malaxation) before oil extraction provokes cell rupture and interactions between cell wall polysaccharides and hydrophilic compounds (phenols, proteins, etc). Oxidation, condensation and/or polymerization reactions by enzymatic or non-enzymatic action provide an important non-carbohydrate polymeric material associated with the cell wall polysaccharides (**Obied, Allen, Bedgood, Prenzler, & Robards, 2005**).

In previous studies by our group we analyzed the pectin material isolated from hydrothermal treated alperujo (160º C/ 30–60 min) (**Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2015a**) and from a gentle heat treated alperujo (50–80º C/1–2 h) (**Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, García, & Fernández-Bolaños, 2015b**). In both cases the pectins presented different and characteristic properties. For "gentle" heating, the pectin-like polysaccharides with high polyphenol content showed a high capacity for binding bile salts and glucose, and a high antioxidant activity. The harsh hydrothermal treatment favored the recovery of pectin with a low molecular weight that could potentially resemble to modified pectin, extracted and modified from numerous source, mainly citrus (**Jackson et al., 2007; Fang et al., 2018**) or apple (**Li et al., 2012**), which are absorbed by the intestinal tract and

have beneficial biologic activities. Increasingly, evidence is linking to these small and short chain molecules of pectin, known as "modified pectin," which act as an inhibitor of the pro-metastasic protein galectin-3 (**Morris, Belshaw, Waldron, & Maxwell, 2013**), induce apoptosis, reduce proliferation and inhibit cell adhesion in numerous cancer cell lines (**Inohara, & Raz, 1994**)(**Jayaram, Kapoor, & Dharmesh, 2015**)(**Maxwell, Colquhoun, Chau, Hotchkiss, Waldron, Morris, & Belshaw, 2015**)(**Pienta et al., 1995**). Although the mechanisms of the anticancer bioactivity of pectins are unknown, interaction with pectins' galactan side-chains was postulated to inhibit the function of the pro-metastatic protein galectin-3, inhibiting their capacity to promote cell adhesion and migration hence preventing tumor growth (**Gao, Zhi, Sun, Peng, Zhang, Xue, Tai, & Zhou, 2013**)(**Gunning, Bongaerts, & Morris, 2008**). Other protein with a carbohydrate recognition domain such galectin-1 has been described as molecule capable of influencing metastasis and play a role in apoptosis and cell growth regulation (**Iurisci et al., 2009**).

According to American Cancer Society, bladder cancer is one of the most common cancers among human population, the fourth in men, but it less common in women, with an estimated 80470 new cases (about 61700 in men and 18770 in women) for 2019 in USA (**American Cancer Society**). Bladder cancer is a complex disease consistent mainly of trasitional cell carcinoma, more than 90%, while squamous cell only represent 1-2% of the cases. It is associated with high morbidity and mortality rates if not treated optimally. Bladder cancer can be describe as superfitial or non-muscule invasive, that are in the trasitional epithelium, or as muscle-invasive, that grow into deeper larger of the bladder wall, and are harder to treat; almost all of squamous cell carcinoma are invasive (**Kanat et al., 2016**). Advances disease is the best treated with sytemic cisplatin-base chemotherapy. There is an intense public and scientific interest in finding other approaches to controlling and treating bladder cancer, resulting in the use of natural substance and/or combination chemotherapy, that could help reduce the dose of cisplatin which would lead to fewer secondary effects.

In this study, the anti-proliferative activity of several polysacaccharide- and polyphenol-rich extracts obtained from steam-treated alperujo at 150º C for 30 min were investigated in four distinct bladder cancer cell lines and compared with the effect of a commercial modified citrus pectin (MCP). Also, the hydrocolloid isolated material was characterized and the antioxidant and the hemagglutination activity were evaluated using in vitro assays. In addition, the secretion of galectin-1 and galectin-3 proteins by exposure of bladder cancer cells to olive extract and modified citrus pectin (MCP) were quantified.

Cisplatin-based combinations represent the standard chemotherapy for patients with metastatic bladder cancer. We hypothesized that pectins would enhance the cytotoxic effect of cisplatin in vitro. Furthermore, we evaluated the combined effect of the olive extracts and MCP with cisplatin in bladder cancer cells.

2. Materials and Methods

2.1. Isolation and purification of olive extracts, named Pectoliv, from alperujo

Five olive extracts (Pectoliv) were isolated from steam-treated alperujo at 150° C for 30 min according to the procedure previously described (**Rubio-Senent, et al., 2015a**). Briefly, samples 1, 3 and 4 were prepared from the fraction released from heat treatment, which was ultra-filtered using a 3 kDa membrane. The fraction with a size greater than 3 kDa was precipitated with 85% ethanol and then purified by ethylenediaminotetracetic acid (EDTA) complexation and dialysis through a 1 kDa membrane, followed by percolation through a strong ionic exchanger resin. The purified extracts are referred to as Pectoliv 1, Pectoliv 3, and Pectoliv 4.

Samples 2 and 5 were prepared from samples 1 and 4, respectively, which were subsequently treated with a chemical hydrolysis at 95° C with 2 N trifluoracetic acid during 3 h in order to remove the neutral sugars and increase the proportion of galacturonan backbone with respect to neutral polysaccharides. Precipitation with 85% ethanol facilitated the precipitation of an insoluble material containing the extracts. The

resulting precipitates were named Pectoliv 2 and Pectoliv 5. (Pectoliv 1→Pectoliv 2)(Pectoliv 4→Pectoliv 5)

Alperujo (olive pomace) was collected directly after the two-phase centrifugal system used in local pomace processing (Oleícola el Tejar, Córdoba, Spain) for the extraction of olive oil during three different times throughout olive season.

2.2. Characterization of the isolated olive extracts

Galacturonan (anhydrogalacturonic acid) was estimated according to the m-hydroxydiphenyl method described by **Blumenkrantz and Asboe-Hansen** (1973) for uronic acids..

Glycosyl compositions were determined by gas chromatography (GC) after their conversion to alditol acetates, and quantified as alditol acetates. Individual neutral sugars were analyzed from duplicate samples with initial trifluoroacetic acid (TFA) hydrolysis (2 N TFA at 121° C for 1 h) prior to reduction, acetylation, and analysis by GC (**Englyst, Wiggins, & Cummings, 1982**). Inositol was used as an internal standard. Calibration was performed with a series of standard sugar solutions of L-rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man) and D-xylose (Xyl). The chromatographic conditions utilized were previously described (**Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández-Bolaños, 2012**).

Total phenolic content was determined by the Folin–Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents (Singleton, & Rossi, 1965). Protein was determined by the micro Kjeldahl method using the multiplication of the total nitrogen content by a conversion factor of 6.25 (Gibson, 1904). The analysis of ash was performed according to the Association of Official Agricultural Chemists procedure (**AOAC, 1990**) .

2.3. Ion exchange chromatography

The method used was described by **Jiménez,Rodríguez, Fernández-Caro, Guillén, Fernández-Bolaños, & Heredia, A.** (2001) with some modification. 5 mL Hi-Trap

Columns (anionic exchanger: Q-Sepharose) from Pharmacia (Uppsala, Sweden) were used. The column was equilibrated in a 0.01 M imidazole-HCl buffer, pH 7. The flow during injection was 1 mL/min. Two steps in the fractionation were made with an increasing buffer concentration of imidazole-HCl from 0.01 to 2.0 M, pH 7. In the first step, a flow of 1 mL/min was employed and neutral oligosaccharides were collected in fractions of 2 mL. In the second step, a flow of 5 mL/min was employed and acid oligosaccharides were collected in fractions of 5 mL. All fractions were assayed for uronic acids and neutral sugar according to the colorimetric method.

2.4. Relative molecular weight determination

The homogeneity and molecular weight distribution of the purified polysaccharide-enriched extracts were estimated by high performance size exclusion chromatography (HPSEC) using two different columns (300 X 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA) in sequence TSKgel GMPWXL (dextran MW<50000 kDa) and TSKgel G3000PWXL (dextran MW<60 kDa) as described previously(Dos-Santos, Jiménez-Araujo, Rodríguez-Arcos, & Fernández-Trujillo, 2011). The system was calibrated with standard dextrans of 252, 110, 70, 40, and 6 kDa, and glucose, using a regression curve.

2.5. *Antioxidant activity by oxygen radical absorbance capacity (ORAC) assay*

The ORAC assay is based upon the inhibition of the peroxylradical-induced oxidation initiated by the thermal decomposition of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH). The reactive oxygen species (ROS) generated from this thermal decomposition quenches the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples were assayed according to **Ou, Hampsch-Woodill, & Prior**, (2001) with minor modifications. Samples were diluted with sodium phosphate buffer (10 mM, pH 7.4) and 25 µL of sample was transferred to a microplate. The blank well received 25 µL phosphate buffer while standards received 25 µL trolox solutions (10–140 µM). Then 150 µL of 1 µM fluorescein was added to all wells. After incubation

(37º C, 15 min), 25 µL AAPH (250 mM) was added to each well to initiate the reaction and a reading taken every 5 min for 90 min (Ex. 485 nm, Em. 538 nm) in a microplate reader (Fluoroskan AscentTM, Thermo ScientificTM). Results were calculated using the difference of areas under the fluorescein decay curve between the blank and the sample as the mean of the three independent experiments and expressed as µmol Trolox equivalents/g sample.

2.6. Bladder cancer cell lines culture

Three cell lines derived from transitional, one superficial or non-muscule invasive (RT112), and two muscule invasive (J82, T24), and one squamous (ScaBER) bladder tumors were obtained from the American Type Culture Collection and grown under standard tissue culture protocols. Cells were grown for no longer than four to six passages and harvested at 75–90% confluence. After harvesting, cell pellets were washed three times in cold PBS and frozen at -20° C for protein extraction. To test inter-assay reproducibility, all experiments were evaluated in triplicate in independent runs.

2.7. Cisplatin and pectin exposure

Cells at a minimum of 70% confluence were exposed to Pectoliv 1-5 extracts (3.1, 6.3, and 12.5 mg/mL), commercial modified citrus pectin (MCP) (PectaSol-C, Econugenics, Santa Rosa, California) (3.1, 6.3, and 12.5 mg/mL) and cisplatin (CDDP) (25 and 50 µM) and observed at different time points for each functional assay. Exposure to each agent was performed individually and combined in a sequential manner.

2.8. Proliferation assay-MTT.

In triplicate, 5 × 10^5 cells/well were seeded in 96-well plates in DMEM containing 10% FBS and incubated for 24 h. Proliferation of bladder cancer cells was determined by measurement of proliferation after 24 h of treatment with Pectoliv 1-5, MCP and cisplatin using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT cell proliferation kit (Roche Diagnostics) was used as

recommended by the manufacturer. The number of viable cells is determined based on the formation of purple formazan crystals by the enzymes in viable cells. After 24 h incubation with the treatment samples, media was removed from the well plates, and 100 µL MTT reagent (50 µg/mL) added per well at 37º C in 5% CO_2. After 4h incubation, the supernatant was removed and 100 µL DMSO added per well to dissolve blue formazan crystals, and the absorbance read at 595 nm. All experiments were evaluated in triplicate in independent runs.

2.9. Protein analysis of galectins in supernatants of bladder cancer cell lines (ELISA assay)

Human bladder cancer cell lines were treated with different Pectoliv extracts or MCP before or after cisplatin treatment for 24 h. Cell supernatants were analyzed by enzyme-inmunoanalyses using galectin-1 and galectin-3 ELISA kits (Calbiochem) and absorbance measured at 450 nm. Protein solutions were analyzed in duplicate in independent runs using Bradford Reagent (Sigma-Aldrich) and absorbance measured at 595 nm. All samples were prepared in triplicate and absorbance measurements taken for each sample in duplicate.

2.10. Hemagglutination assay

A semi-quantitative agglutination assay was carried out by incubating the trypsinized, glutaraldehyde-fixed rat erythrocytes (40 µL, 1.6 mg, 4% w/v in PBS buffer) in the presence of increasing concentrations of Pectoliv and MCP in 96-well U-bottom microplates for 1 h at room temperature. Agglutinating activity was categorized as negative or positive for each well. Galectin-3 (0.5 µg/well) was added to erythrocytes as a negative control and incubations of erythrocytes with no sample or no galectin-3 added were used as a positive control.

2.11. Statistical analysis

Results were expressed as mean values ± standard deviations. STATGRAPHICS® plus software was used for statistical analysis. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) and the LSD method. A p-value< 0.05 was considered significant.

3. Results

3.1. Obtention and chemical characterization of olive extracts

The isolation and purification of olive extracts Pectoliv 1, Pectoliv 2 and Pectoliv 3 was carried out using three samples of different alperujo obtained from hydrothermal treatments at 150º C for 30 min, following a procedure similar to that used for the isolation and purification of extracts rich in pectic polysaccharides as previously reported (**Rubio-Senent et al 2015**a). The composition of this isolated material (**Table 1A**) was different from that reported in our previous work, probably due to the wide variability of alperujo samples (variety of olive, state of ripening, influence of season) and the different conditions of treatment. The hydrothermal treatment of alperujo generated a material whose total sugar content (the sum of the percentage of uronic acid and neutral sugar) was 65–75%, with a very high ratio of neutral and acidic polysaccharides (2.5 to 10) in comparison with the previously reported extract (1.1 to 2.6); whereas the phenolic compounds presented values of 17–24%, more than double that reported for the material isolated in previous work (**Rubio-Senent et al., 2015a**). The composition of purified extracts was similar to a polymeric fraction isolated from olive oil mill wastewater, a by-product of three phase process of olive extraction (**Capasso, de Martino, & Arienzo, 2002**), composed of phenols or melanin, and polysaccharides, albeit with a lower protein and inorganic substance content. The phenolic compounds seem to be responsible for the brown color of the extracts. These pigments from olive mill waste were identified as catechol-melanin macromolecules derived from the polymerization action of the phenolic compounds linked to polysaccharides, protein and fatty acids (**Obied et al., 2005**).

Table 1. Chemical composition of Pectoliv extracts of steam-treated olive by-product (alperujo) and commercial modified citrus pectin (MCP) expressed as g/100 g sample. Glycosyl residue composition analysis (% molar ratio). A) Pectoliv samples 1, 3, and 4; B) Pectoliv samples 2 and 5 and MCP. The data shown are mean ± standard deviation. Rha: rhamnose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; UrA: uronic acids.

	Pectoliv		
A)	**1**	**3**	**4**
Uronic acid	5.80 ± 1.05	16.48 ± 1.72	18.46 ± 1.96
Neutral sugar	59.91 ± 3.24	59.20 ± 15.10	46.91 ± 1.93
Phenol	23.92 ± 3.33	16.80 ± 0.35	22.75 ± 1.67
Protein	0.99 ± 0.26	3.34 ± 0.13	4.16 ± 0.20
Ash	2.30 ± 0.22	1.73 ± 0.14	3.22 ± 0.19
	92.92	97.56	95.50
		% molar	
Rha	5.24 ± 0.15	3.68 ± 1.01	5.03 ± 0.17
Ara	20.66 ± 1.37	23.75 ± 6.30	29.19 ± 1.33
Xyl	26.37 ± 1.92	26.20 ± 6.46	7.05 ± 0.31
Man	7.34 ± 0.06	3.56 ± 1.11	4.37 ± 0.13
Gal	22.21 ± 0.83	15.79 ± 3.69	19.04 ± 0.69
Glc	8.88 ± 0.26	6.12 ± 1.63	7.96 ± 0.31
UrA	8.80 ± 1.67	25.63 ± 2.67	28.71 ± 3.05

B)	MCP	Pectoliv 2	Pectoliv 5
Uronic acid	51.52 ± 7.78	11.14 ± 0.59	45.43 ± 6.53
Neutral sugar	5.24 ± 0.07	15.30 ± 1.59	8.64 ± 1.09
Phenol	0.15 ± 0.01	72.30 ± 1.73	19.16 ± 1.02
Protein	3.69 ± 0.06	0.52 ± 0.01	8.13 ± 1.73
Ash	20.33 ± 0.19	1.95 ± 0.20	7.02 ± 0.18
	80.93	101.21	88.39
		% molar	
Rha	1.69 ± 0.11	15.42 ± 2.62	2.94 ± 0.39
Ara	1.08 ± 0.14	1.14 ± 0.07	1.08 ± 0.51
Xyl	0.49 ± 0.04	1.97 ± 0.13	1.23 ± 0.26
Man	0.56 ± 0.07	31.25 ± 2.51	7.57 ± 0.54
Gal	4.67 ± 0.08	1.29 ± 0.00	0.72 ± 0.10
Glc	0.63 ± 0.01	6.55 ± 0.44	2.37 ± 0.12
UrA	90.77 ± 0.35	42.07 ± 0.27	83.96 ± 13.33

Analysis of the neutral sugar composition of the isolated extracts (**Table 1A**) showed the presence of Ara, Gal, and Xyl in high proportions. Minor amounts of Rha, Man and Glc were also present in the three samples. Steam treatment promoted the cleavage of the galacturonic backbone and the arabinan and galactan side chains, together with other polysaccharides differing in their sugar composition, and probably originating from arabinoxylan, glucomannan or xyloglucan, which were previously described as constituents of olive pulp (**Jiménez et al., 1994**). The presence of these neutral polysaccharides strongly bound to the acid sugar chain and to polyphenols was demonstrated by anion exchange chromatography on Q-Sepharose, where the color eluted represented an important proportion of neutral and acidic polysaccharides with 2.0 M imidazole buffer (data not shown). This observation confirmed the presence of an aggregate similar to that found by **Capasso et al**. (2002) in olive oil mill waste water.

The samples Pectoliv 1 and 4 were transformed by acid hydrolysis in order to reduce the ratio of neutral and acidic polysaccharides and to obtain a more similar

composition to commercial modified citrus pectin, a known inhibitor of galectin-3. Chemical analysis of Pectoliv 2 and 5 compared with Pectoliv 1 and 4 showed a substantial loss of neutral sugars and an increase of uronic acid, of 1.92 or 2.46 times, respectively (**Table 1B**). However, the phenolic compounds in Pectoliv 2 increased from 23.9 to 72.3 mg/ 100 g extract, while in Pectoliv 5 there was practically no change, although the values were very high in comparison with MCP, in which phenolic compounds were practically absent. In this case and following the indications of the U.S Pharmacopeia that define pectin as having not less than 74 % of galacturonic acid, the Pectoliv 5 with a 84 % of uronic acid similar to MCP (91 %), was the only sample that could consider modified pectin from alperujo, although the other samples could be consider pectin-like polysaccharides and all with a high proportion of phenols associated.(**Table 1B**).

3.2 ***Determination of molecular size distribution***

The relative molecular size of the five samples of Pectoliv was performed (**Figure 1**). Pectoliv 1 had an approximate molecular size distributed in the range of 110 KDa and 2 KDa, while Pectoliv 3 and 4 ranged approximately between 500 KDa and 2 KDa. In the three samples, the highest proportion of compounds eluted in the zone with an average size between 70 KDa and 2 KDa. The reduction of the neutral polysaccharides after acid hydrolysis was reflected in the relative molecular size of Pectoliv 2 and 5, with a decrease in the intensity of zones corresponding to high molecular weight eluates, indicating a breakage of the polysaccharide chains into smaller ones. Both extracts eluted between 40 and 2 KDa, with a maximum peak close to 16 KDa, similar to the molecular size of modified citrus pectin (MCP) with 3-13 kDa according the specifications, which theoretically would be consider systemically bioactive.

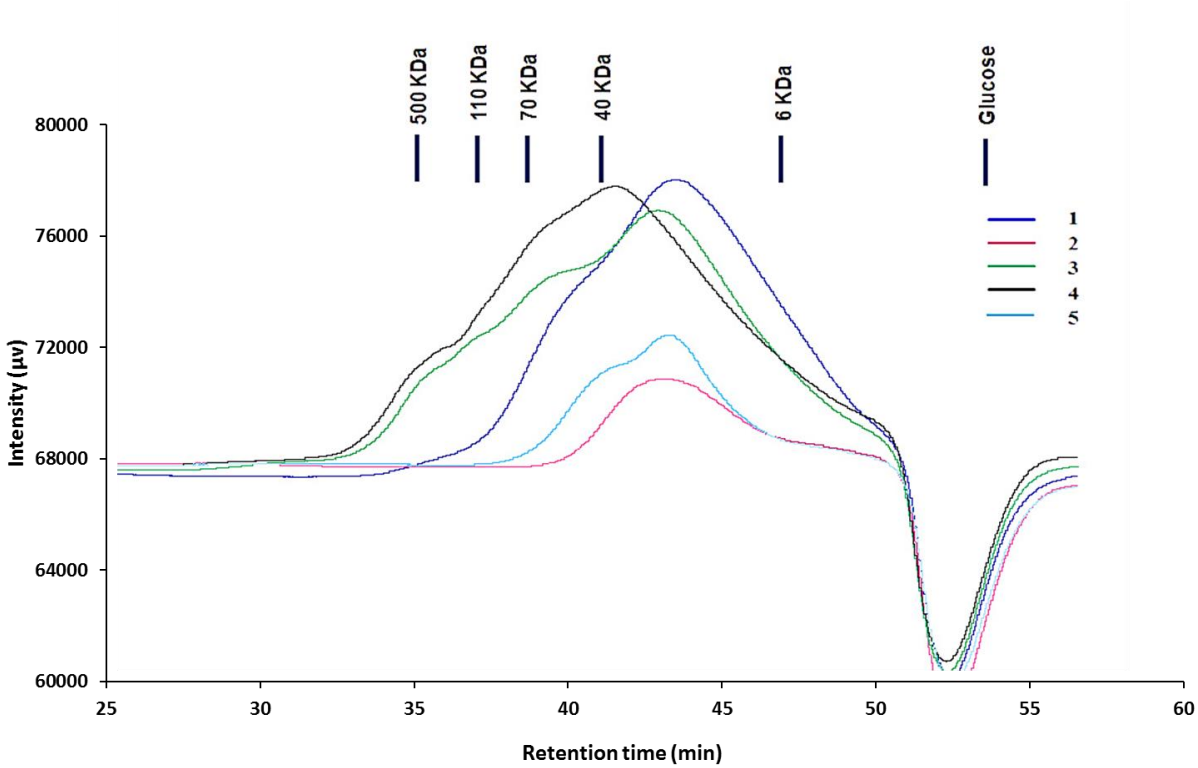


Figure 1. Refractive index elution profiles from the Pectoliv 1-5 extracts obtained by high performance size exclusion chromatography with two TSK gel columns (GMPWXL and G3000PWXL) placed in a series. Number above the peaks represent molar masses of dextran.

3.3. Antioxidant activities of Pectoliv extracts

The five Pectoliv extracts showed a noticeable effect on oxygen radical absorbance capacity (ORAC) (**Figure 2**). All the extracts displayed higher antioxidant capacity than the MCP, which exhibited very low activity. The compositional differences could explain this capacity due to the content of polyphenols observed in all extracts. Of the five extracts, Pectoliv 2 showed the highest antioxidant activity, coinciding with the highest content of polyphenols (72%). However, for the other extracts, there was no clear correlation between the phenol content and antioxidant activity. It is probably that the phenolic composition or the form to linking to the polysaccharides will be some different for each extract. The antioxidant activity values for Pectoliv extracts (774–2581 µmol Trolox/g extract) were much higher than the total antioxidant capacity of

the most common European fruit and vegetable varieties, which are in the range of 16 to 208 µmol Trolox/g dry matter and include the antioxidant capacity of extractable polyphenols and macromolecular hydrolyzable polyphenols (**Pérez-Jiménez, & Saura-Calixto, 2015**). The values were in the order of the soluble dietary fiber of the tropical fruit açai (603 µmol Trolox/g extractable polyphenols) plus the insoluble dietary fiber (209 µmol Trolox/g extactable and hydrolyzable polyphenol) (**Do Socorro et al., 2011**). Therefore, the hydrocolloid extracts obtained from steam-treated alperujo with associated phenolic compounds can be considered as an important source of antioxidants.

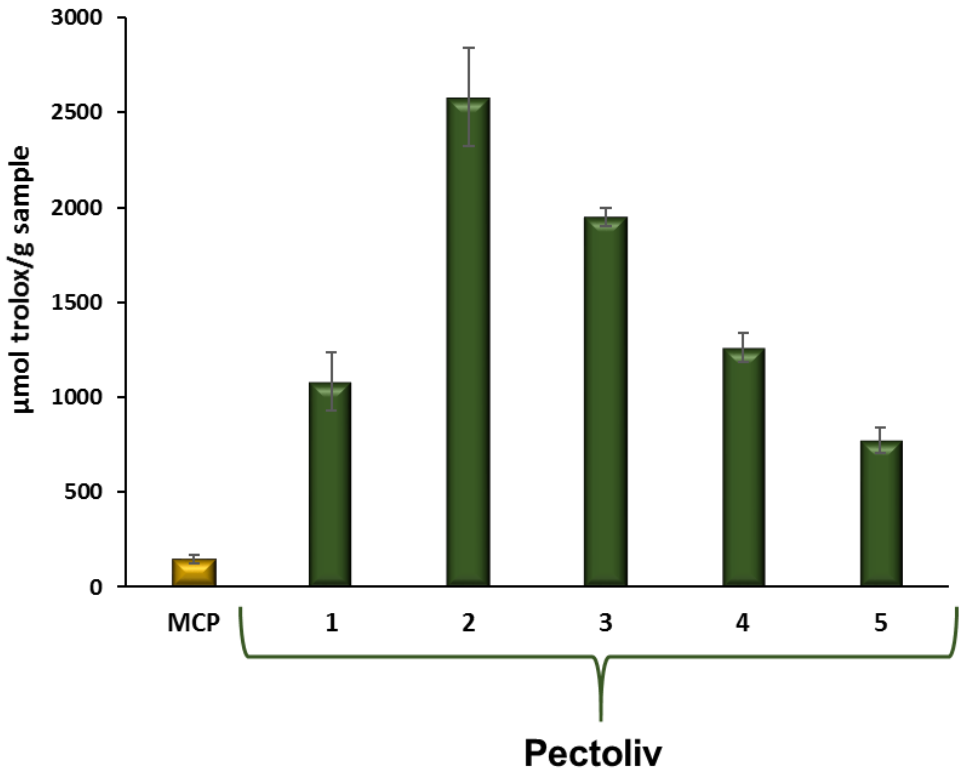


Figure 2. Antioxidant capacity of Pectoliv 1-5 extracts compared to those for commercial citrus pectin and commercial modified citrus pectin (MCP). Oxygen radical capacity (ORAC) is expressed as µMol Trolox/g extract. Each bar is the average value of three replicates. The error bars represent standard deviations (n = 3).

3.2. Antiproliferative effect on bladder cancer cells

We evaluated the functional effect of the five Pectoliv extracts at 12.2 mg/mL on four different human bladder cancer cell lines and compared this with the effect on proliferation of a commercial MCP at the same concentration or cisplatin at 25 and 50 µM. Pectoliv exposure produced an important inhibition of proliferation after 24 h in all the bladder cancer cell lines studied, as observed by the MTT assay (**Figure 3**). Moreover, the inhibitory effect was higher with respect to the commercial MCP for all cancer cell lines. MCP was recently reported to suppress T24 and J82 bladder cancer cell proliferation after 72 h of treatment at 5–20 mg/mL by sulforhodamine B (SRB) assay (**Fang et al., 2018**). The squamous SCaBER cells were the most sensitive to Pectoliv extracts, with an 80% reduction in cell proliferation, whereas RT112 cells were the least sensitive with a reduction of 20–40%. The olive-derived extracts had a similar or even more effective antiproliferative effect on SCaBER cells than cells treated with cisplatin, a common chemotherapeutic agent. It is interesting remark that none of the Pectoliv extracts shown inhibition the growth of confluents cultures of human colon carcinoma Caco-2 cells lines (data not shown). When these Caco-2 cells are allowed to grow in confluent cultures they are differentiated and transformed in healthy epithelial cells (**Girón-Calle et al., 2004**)(**Artursson, Palm, & Luthman, 2001**). Further proliferation analyses were performed in these two cell lines comparing each Pectoliv extract versus MCP at increasing concentrations. These studies showed that the olive-derived extracts affected cell proliferation more considerably than MCP in both the more sensitive (**SCaBER, Figure 4A**) and less sensitive (**RT112, Figure 4B**) cell lines. This higher efficacy could be due to the presence of phenols, which were practically absent in the MCP. However, despite the different amounts of phenols in the extracts, the decreases of cell proliferation in SCaBER cells were very similar; whereas in RT112 cells, the Pectoliv 2 sample, with a higher value of phenolic compounds (72%), showed a somewhat lower inhibition of proliferation. Furthermore, no relationship was observed between the different ratios of neutral/acid polysaccharides and the comportment of Pectoliv 1, 3, and 4 with respect to extracts 2 and 5. Thus, structure-function studies on

the bioactivity of the Pectoliv extracts are required for a better understanding of their anticancer effects. However, the high antioxidant activity and the presence of an important amount of phenolic compounds found in the extracts could be related to the antiproliferative activity. These findings are in agreement with those of other authors who published similar observations with different degrees of potency depending on the type of cancerous cell line and antioxidant extract studied (**Seeram et al., 2006)(Tow, Premier, Jing, & Ajlouni, 2011**).

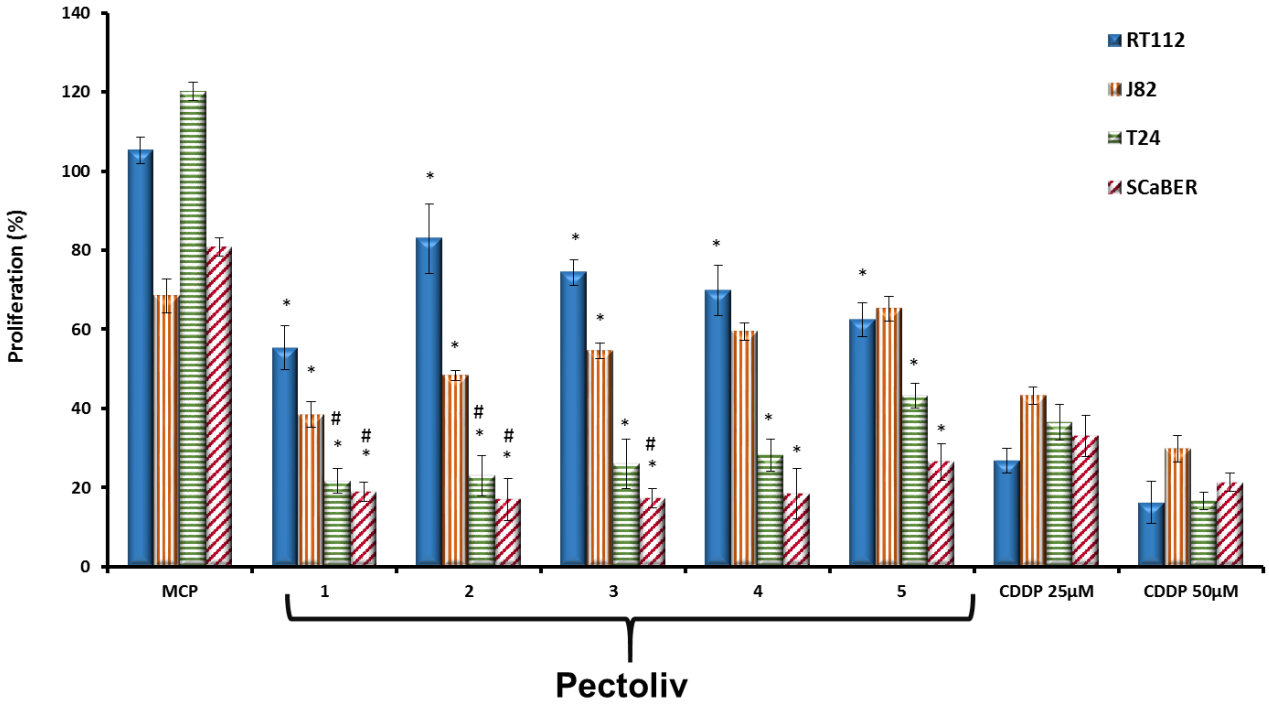


Figure 3. Effect of Pectoliv extracts and MCP at 12.5mg/mL or cisplatin (CDDP) ar 25 and 50 µM on the proliferation of human bladder cancer cell lines (RT112, J82, T24 and SCaBER) by the MTT assay. Cells were treated with Pectoliv and MCP at the indicated concentration for 24 h at 37º C. proliferation (%) is expressed as relative percentages to cell control. * indicate statistical significance respect MCP and # indicate statistical significance respect CDDP 25 µM ($p<0.05$). Bars represent mean ± SD.

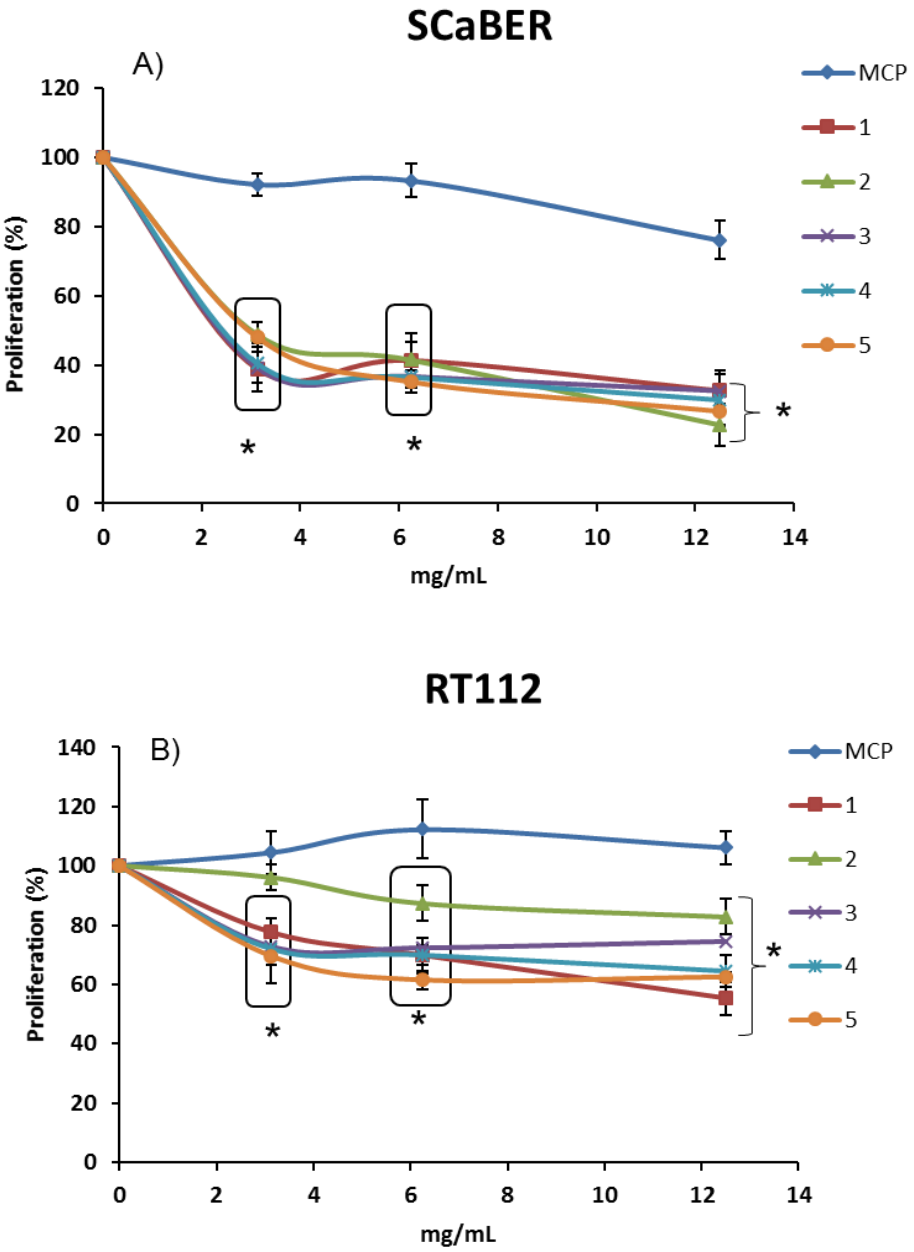
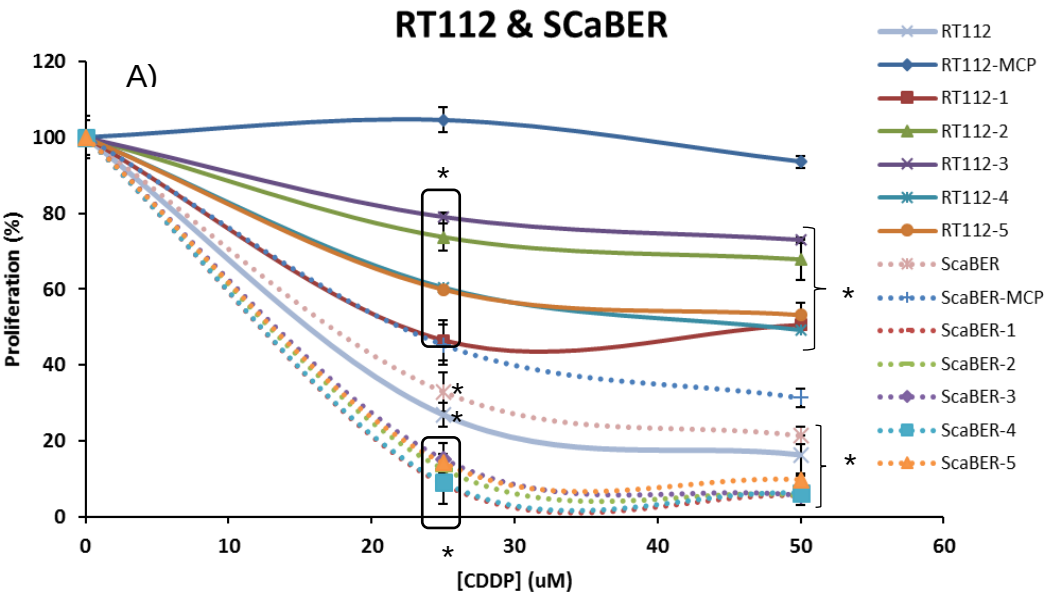


Figure 4. Anti-proliferative activity of Pectoliv or MCP at three concentrations (3.1, 6.3, and 12.5 mg/mL) for 24 h on A) SCaBER and B) RT112 cells. Cell proliferation was analyzed by MTT assay. Proliferation (%) is expressed as relative percentages to cell control. Asterisks indicate statistical significance respect MCP ($p<0.05$). Bars represent mean ± SD.

We also studied the combined treatment of Pectoliv extracts or MCP and other therapeutic agents, such as cisplatin, on the SCaBER and RT112 bladder cancer cells. When cells were treated with Pectoliv extracts 1-5 in combination with cisplatin at two different concentrations, 25 and 50 µM, SCaBER remained the most sensitive cell line, causing an additive effect on cisplatin's ability to inhibit cell proliferation (**Figure 5A**). However, in the case of the combination of cisplatin-MCP/Pectoliv (Cisplatin, (25-50 µM) and MCP/Pectoliv at 12.5 mg/mL), SCaBER cells had higher proliferation than cells treated with cisplatin alone. For RT112 cells, cisplatin did not exert an additional cytotoxic effect when treated in combination with Pectoliv extract or MCP, and remain above of treated with cisplatin alone (**Figures 5B, 5C**). Exposure to cisplatin both before and after treatment with Pectoliv extracts or MCP enhanced cisplatin's cytotoxic effect in sensitive cells (**Figure 5B**). In RT112, the least sensitive cell line, the exposure of cells to Pectoliv and MCP before cisplatin protected the cells from the cytotoxic effect of cisplatin (**Figure 5C**).



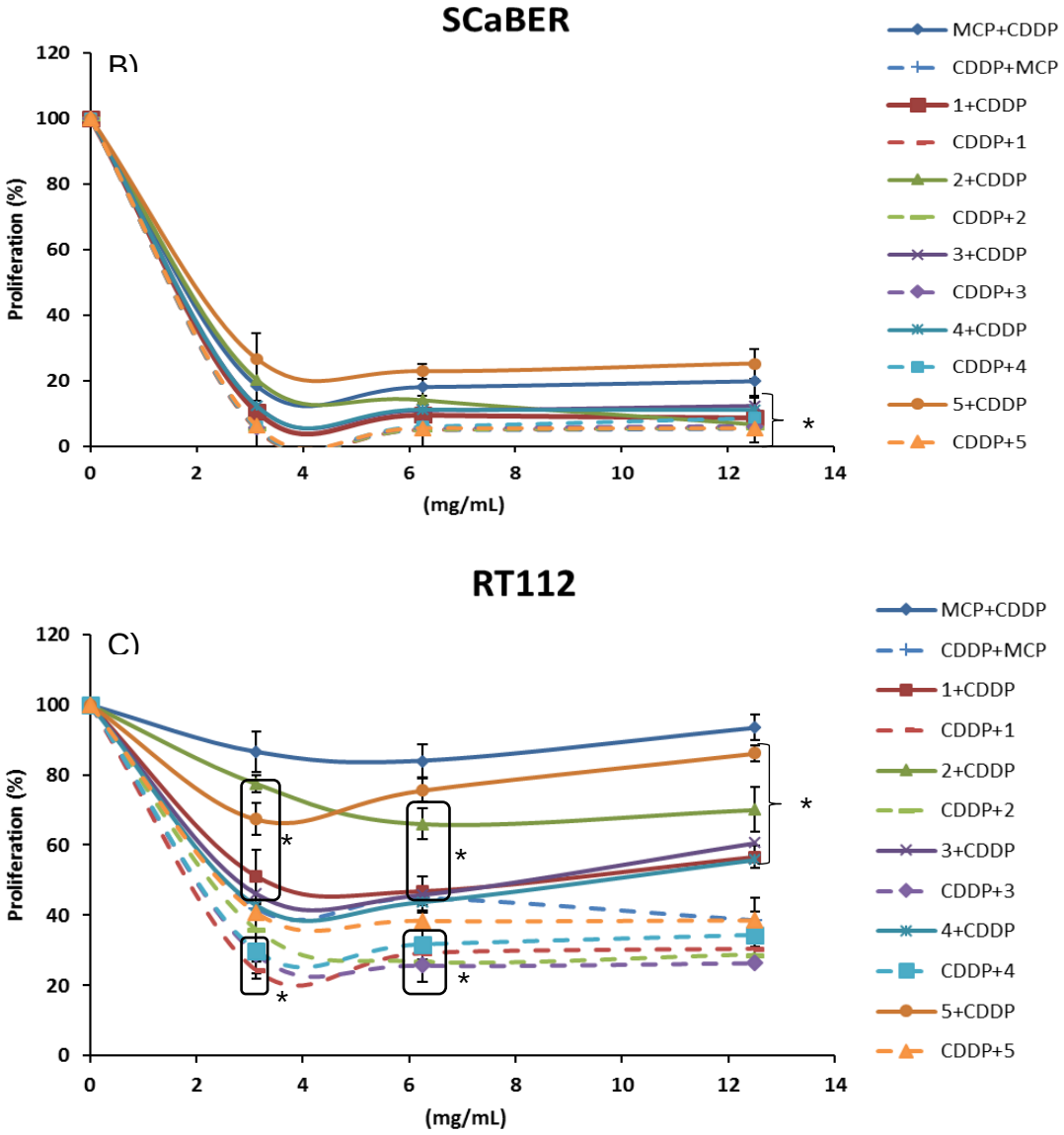


Figure 5. Anti-proliferative effect of Pectoliv-cisplatin and MCP-cisplatin combinations on RT112 and SCaBER bladder cancer cell lines. (A) The cells were treated with Pectoliv and MCP at 12.5 mg/mL and cisplatin (25 and 50 µM) for 24 h (simultaneous treatment). RT112 (B) and SCaBER cells (C) were pre-treated with Pectoliv or MCP (3.1, 6.3, and 12.5 mg/mL) for 24 h and then treated with cisplatin (50 µM) for another 24 h or viceversa. Cell proliferation was analyzed by MTT assay. Proliferation (%) is expressed as relative percentages to cell control. Asterisks indicate statistical significance respect MCP ($p<0.05$). Bars represent mean ± SD.

3.3. Galectin-1 and galectin-3 secretion upon exposure to Pectoliv extracts and cisplatin

An elevated expression of galectin-1 and galectin-3 has been reported in bladder cancer cells (**Canesin, Gonzalez-Peramato, Palou, Urrutia, Cordón-Cardo, & Sánchez-Carbayo, 2010**)(**Cindolo et al., 1999**). Galectin-1 and -3 are β-galactose-binding proteins involved in different steps of tumor progression (regulation of proliferation, apoptosis, cell cycle, metastasis and angiogenesis) and are potential targets for anti-cancer therapy (**Cindolo et al., 1999**)(**Bieg ,Sypniewski, Nowak, & Bednarek, 2018**). In the case of galectin-1, its secretion by tumor cells suppresses the tumor immune response through the induction of apoptosis of activated T cells (**Rubistein et al., 2004**). Cytoplasmic galectin-3 is a well-known anti-apoptotic (**Jackson, 2007**). In addition, the targeting of galectin-3 by MCP has recently been demonstrated in T24 and J82 bladder cancer cells (**Fang et al., 2018**). Thus we explored the secretion of galectin-1 and galectin-3 proteins in RT112 and SCaBER cells when exposed to Pectoliv extracts and MCP. Also the secretion of both proteins in the presence or absence of cisplatin, and sequentially in combination were measured by ELISA in cell supernatants. Our results show that Pectoliv treatment slightly suppress galectin-1 and -3 protein expression, whereas MCP significantly reduced the expression of galectin-1 (**Figure 6A, B**). The levels of galectin-1 and -3 only increased when SCaBER cells were treated with Pectoliv-1 or, in the case of RT112 cells, treated with Pectoliv-2, the extract with the highest phenolic concentration.

The expression of galectin-1 and -3 with the combined effect of Pectoliv and MCP with cisplatin, showed that cisplatin alone significantly increased the expression levels of galectin-1 and -3 in the SCaBER cell line only (**Figure 6C, D**). These data are similar to those reported by previous studies, which showed that cisplatin effectively increases galectin-3 expression in ovarian cancer cells (**Fang et al., 2018**) and K562 leukemia cells (**Cheng, et al., 2011**), protecting cells from apoptosis. However, the exposure of Pectoliv extracts and MCP significantly reduced the expression of galectin-3 in SCaBER cells treated with cisplatin for 24 h, with a higher reduction observed when the cells were treated with cisplatin before Pectoliv and MCP treatment. Similar results were observed for the expression of galectin-1 when SCaBER cells were exposed to Pectoliv or MCP before cisplatin, although surprisingly,

in the case of RT112, the secretion of galectin-1 increased upon exposure to Pectoliv and MCP after cisplatin. Therefore, our results show that Pectoliv and MCP have a similar comportment, causing a decrease of the expression of galectin-3 (an anti-apoptotic protein). In addition we demonstrated that both Pectoliv and MCP may sensitize bladder cancer cells to cisplatin, reducing considerably the expression of galectin-3. Also, the inhibition of galectin-1 expression by MCP, and to a lesser extent by Pectoliv, is interesting since galectin-1 constitutes an important mechanism of tumor-immune escape due to the apoptosis of T cells and its inhibition could potentially be targeted as an effective immune response against tumor cells, as proposed by the group of Rabinovich (**Rubistein et al., 2004**).

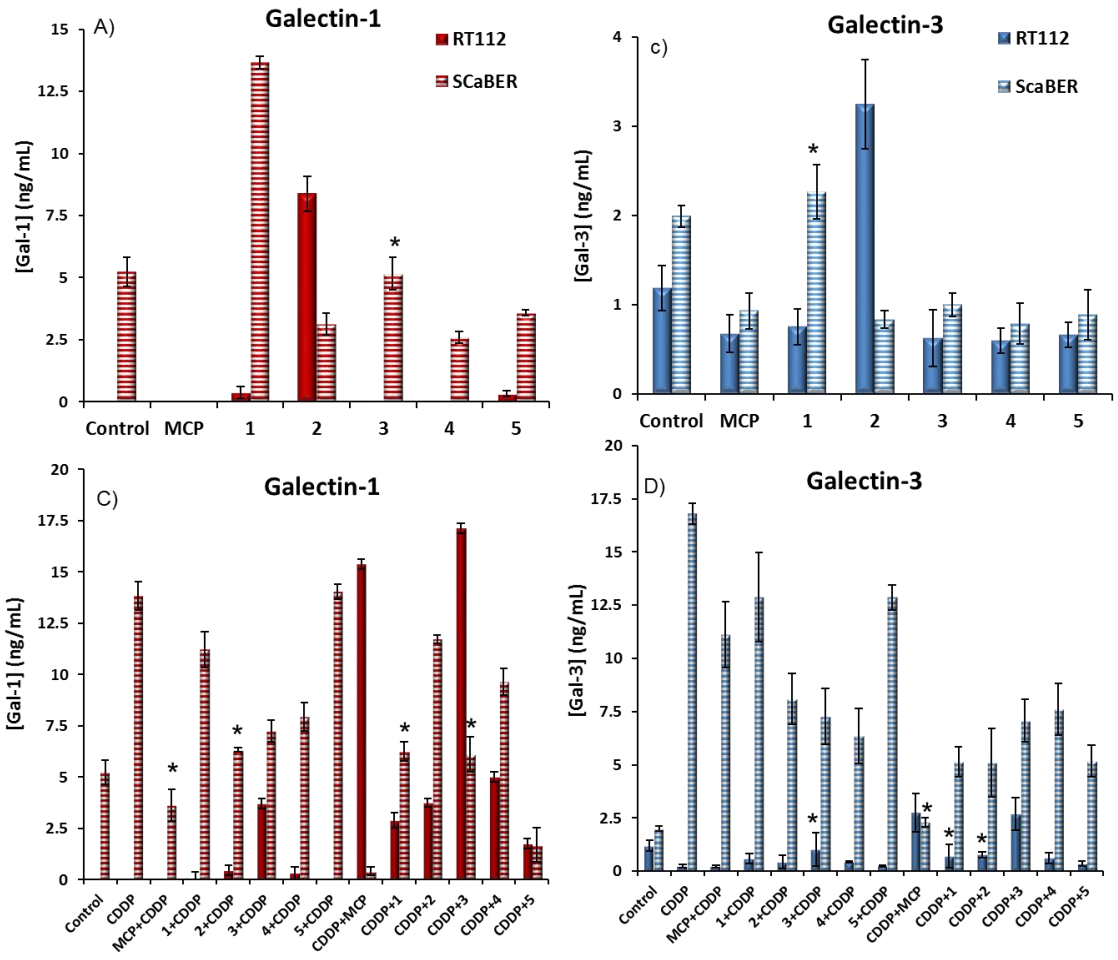


Figure 6. Effect of Pectoliv, MCP and cisplatin on the expression of galectin-1 and galectin-3 in RT112 (less sensitive) and SCaBER (more sensitive) bladder cancer cells. (A, B) The cells were treated for 24 h with Pectoliv and MCP (12.5 mg/mL) and with (C, D) cisplatin (25 µM) for 24 h before and after with Pectoliv and MCP (12.5 mg/mL) for another 24 h. At the protein level, the expression was assessed by ELISA assay. The data are shown as mean ± SD of triplicate experiments. Asterisks indicate not statistical significance respect cell control ($p<0.05$).

3.4. Inhibition of galectin-3 mediated hemagglutination

The galectin inhibitory activity of the five Pectoliv extracts isolated from olive by-products and commercial MCP were evaluated using the hemagglutination assay.

The minimum inhibitory concentration (MIC) of pectin required to inhibit the galectin-3 mediated agglutination of red blood cells was determined and the results were compared with a negative control of galectin-3 added to erythrocytes; incubations of erythrocytes with no sample or no galectin-3 were used as positive control. In the assays, MCP inhibited agglutination at an MIC of 32 mg/mL, whereas Pectoliv extracts showed a much more potent agglutination inhibition, with MICs of 4–8 mg/mL (**Figure 6**). The potential inhibition mechanism for Pectoliv and MCP could be different: In the case of MCP, the binding of galectin-3 to β-D-galactoside residues present on metastasic cancer cells may be blocked (**Sathisha, Jayaram, Harish Nayaka, & Dharmesh, 2007**). In contrast, the hemagglutination inhibitory activity of Pectoliv could be attributed to the interactions of polyphenols present in the hydrocolloid extracts, which include in their molecular structure both aromatic rings and hydroxyl groups, with the galectin-3 protein preventing the agglutination of lectin.

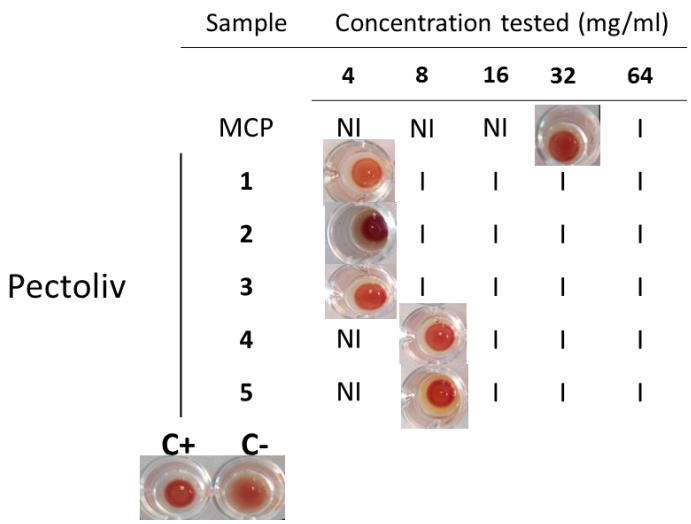


Figure 7. The inhibitory activity on galectin-3 mediated erythrocyte agglutination (inhibition, I and non-inhibition, NI) of Pectoliv extracts and MCP at different concentrations. Positive control (C+): incubations of erythrocytes with no sample or galectin-3; negative control (C-): galectin-3 added to erythrocytes.

4. Conclusions

Our study shows that Pectoliv extracts obtained from thermally treated alperujo are hydrocolloids with a high polyphenol content. They are constituted of pectin-like polysaccharides with different side chains made up of neutral sugars that are strongly bound to the phenol polymeric component and form an aggregate of a relatively low molecular size. In previous work, Pectoliv extracts showed a high capacity for binding bile acid and glucose, implying their beneficial ability to decrease cholesterol levels and delay the intestinal absorption of glucose (**Rubio-Senent et al., 2015a**). In this paper we demonstrate that Pectoliv extracts show a high antioxidant capacity and an important suppression of bladder cancer cell proliferation in vitro. Moreover, the sequential addition of olive-derived extracts enhanced the cytotoxic effect of cisplatin in bladder cancer, suggesting the potential usefulness of these extracts as an adjunct agent for cisplatin chemotherapy. We also found that cotreatment of Pectoliv and commercial MCP was associated with decreased expression of galectin-1 (a protein that induces the apoptosis of T-cells, conferring immune privilege to tumor cells) and galectin-3 (an anti-apoptotic protein). This decrease was particularly prominent when the bladder cancer cells were exposed to Pectoliv and MCP before or after cisplatin treatment, which itself increase the expression of galectin- 1 and -3 expression and protects cell from apoptosis. Our finding confirms that Pectoliv significantly inhibited the agglutination of erythrocytes and could potentially be developed as a novel galectin-3 inhibitor. These results clearly indicate that Pectoliv extracts obtained from the by-product of olive oil extraction could be a good source of natural antioxidants with significant antiproliferative efficacy against bladder cancer cells. Further chemical and biochemical investigations on the elucidation of antiproliferative and antimetastasic mechanisms are currently underway in our labs. Moreover, certainly future research based in vivo tests and clinical studies are required to attest the promising results found.

Abbreviations: AOAC, Official Methods of Analysis; Ara, L-arabinose; CDDP, Cisplatin; UrA, Uronic acid; EDTA, Ethylenediaminotetracetic acid; FBS, Fetal Bovine Serum; Gal, D-galactose; Glc, D-glucose; HPSEC, High Performance Size Exclusion Chromatography; Man, D-mannose; MCP, Modified Citrus Pectin; MIC, Minimum Inhibitory Concentrations; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, Phosphate Buffered Saline; Rha, L-Rhamnose; TFA, Trifluoroacetic acid; Xyl, D-xylose

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Conflict of interest

The authors have no conflicts of interest to declare.

References

American Cancer Society.https://www.cancer.org/cancer/bladder-cancer/about/key-statistics.html. Accessed 13 March 2019.

Artursson, P., Palm, K., & Luthman, K. (2001). Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Advanced Drug Delivery* Reviews, *46*, 27-43.

Bieg, D., Sypniewski, D., Nowak, E., & Bednarek, I. (2018). Morin decreases galectin-3 expression and sensitizes ovarian cancer cells to cisplatin. A*rchives of Gynecology and Obstetrics,* 1-14.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New Method for Quantitative Determination of Uronic Acids. *Analytical Biochemistry,, 54* (2), 484–489.

Canesin, G., Gonzalez-Peramato, P., Palou, J., Urrutia, M., Cordón-Cardo, C., & Sánchez-Carbayo, M. (2010). Galectin-3 Expression Is Associated with Bladder Cancer Progression and Clinical Outcome. *Tumor Bioogy, 31* (4), 277–285.

Capasso, R., de Martino, A., & Arienzo, M. (2002). Recovery and Characterization of the Metal Polymeric Organic Fraction (Polymerin) from Olive Oil Mill Wastewaters. *Journal of Agricultural and Food Chemistry, 50*, 2846-2855.

Cheng, Y.-L., Huang, W.-C., Chen, C.-L., Tsai, C.-C., Wang, C.-Y., Chiu, W.-H., … Lin, C.-F. (2011). Increased galectin-3 facilitates leukemia cell survival from apoptotic stimuli. *Biochemical and Biophysical Research Communications, 412*(2), 334–340.

Cindolo, L., Benvenuto, G., Salvatore, P, Pero, R., Salvatore, G., Mirone, V., … Chiariotti, L. (1999). Galectin-1 and galectin-3 expression in human bladder transitional-cell carcinomas. *Interantional Journal of Cancer,* 84(1), 39–43.

Coimbra, M. A., Cardoso, S. M., & Lopes-da-Silva, J. A. (2010). Olive pomace, a source for valuable arabinan-rich pectic polysaccharides. *Topics in Current Chemistry,. 294,* 129–141.

Do Socorro, M., Rufino, S. M., Pérez-Jiménez, J., Arranz, S., Alves, R. E., De Brito, E. S., Oliveira, M.S.P., & Saura-Calixto, F. (2011). Açaí (Euterpe oleraceae) "BRS Pará": A tropical fruit source of antioxidant dietary fiber and high antioxidant capacity oil.

Food Research International, 44, 2100-2106.

Englyst, H., Wiggins, H. S., & Cummings, J. H. (1982). Determination of the Non-Starch Polysaccharides in Plant Foods by Gas-Liquid Chromatography of Constituent Sugars as Alditol Acetates. *The Analyst, 107* (1272), 307.

Fang, T., Liu, D., Ning, H., Dan Liu, Sun, J., Huang, X., … Huang, R. (2018). Modified citrus pectin inhibited bladder tumor growth through downregulation of galectin-3. *Acta Pharmacologica Sinica, 39,* 1885-1893.

Gao, X., Zhi, Y., Sun, L., Peng, X., Zhang, T., Xue, H., Tai, G., & Zhou, Y. (2013). The Inhibitory Effects of a Rhamnogalacturonan I (RG-I) Domain from Ginseng Pectin on Galectin-3 and Its Structure-Activity Relationship. *Journal of Biological Chemistry, 288* (47), 33953–33965.

Gibson, R. B. (1904). The determination of nitrogen by the Kjeldahl method. *Journal of the American Chemical Society,26* (1), 105–110.

Giron-Calle, J., Vioque, J., del Mar Yust, M., Pedroche, J., Alaiz, M., & Millan, F. (2004). Effect of chickpea aqueous extracts, organic extracts, and protein concentrates on cell proliferation. *Journal* of Medicinal *Food*, *7(2*), 122-129.

Gunning, A. P., Bongaerts, R. J. M., & Morris, V. J. (Recognition of Galactan Components of Pectin by Galectin-3. *The FASEB Journal, 23* (2), 415–424.

Inohara, H., & Raz, A. (1994). Effects of Natural Complex Carbohydrate (Citrus Pectin) on Murine Melanoma Cell Properties Related to Galectin-3 Functions. *Glycoconjugate Journal. 11* (6), 527–532.

Iurisci, I., Cumashi, A., Sherman, A.A., Tsvetkov. Y.E., Tinari, N., Piccolo, E., D'Egidio, M., et al. (2009). Synthetic inhibitors of galectin-1 and -3 selectively modulate homotypic cell aggregation and tumor cell apoptosis. Anticancer Research, *29*(1):403-10.

Jackson, C. L,; Dreaden, T. M., Theobald, L. K., Tran, N. M., Beal, T. L., Eid, M., Gao, M. Y., Shirley, R. B., Stoffel, M. T., Kumar, M. V., et al. (2007). Pectin Induces Apoptosis in Human Prostate Cancer Cells: Correlation of Apoptotic Function with Pectin Structure. *Glycobiology*, *17* (8), 805–819.

Jayaram, S.,Kapoor, S., & Dharmesh, S. M. (2015). Pectic Polysaccharide from Corn (Zea Mays L.) Effectively Inhibited Multi-Step Mediated Cancer Cell Growth and Metastasis. *Chemico-Biological.Interaction, 235*, 63–75.

Jiménez, A., Guillén, R., Fernández-Bolaños, J., & Heredia, A. (1994). Cell Wall Composition of Olives. *Journal of Food Science, 59(6),* 1192-1196.

Kamat, A.M., Hahn, N.M., Efstathiou, J.A., Lerner, S.P., Malmström, P.U., Choi, W., Guo, C.C., Lotan, Y., & Kassouf, W. (2016). Bladder cancer . *Lancet , 388*, 2796–810.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, Characterization and Isolation of Neutral and Pectic Oligosaccharides with Low Molecular Weights from Olive by-Products Thermally Treated. *Food Hydrocolloids, 28* (1), 92–104.

Li, Y., Liu, L., Niu, Y., Feng, J., Sun, Y., Kong, X., Chen, Y., Chen, X., Gan, H., Cao, S., Mei, Q. (2012). Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitisassociated colon cancer: role of galectin-3 and apoptosis in cancer prevention. European journal of nutrition, *51*, 107-17.

Maxwell, E. G., Colquhoun, I. J., Chau, H. K., Hotchkiss, A. T., Waldron, K. W., Morris, V. J., & Belshaw, N. J. (2015). Rhamnogalacturonan I Containing Homogalacturonan Inhibits Colon Cancer Cell Proliferation by Decreasing ICAM1 Expression. *Carbohydrates Polymers, 132*, 546–553.

Morris, V. J., Belshaw, N. J., Waldron, K. W., & Maxwell, E. G. (2013). The bioactivity of modified pectin fragments. *Bioactive Carbohydrates andDietary Fibre*, 1, 21-37

Obied, H. K.,Allen, M. S., Bedgood, D. R., Prenzler, P. D., & Robards, K. (2005). Investigation of australian olive mill waste for recovery of biophenols. *Journal of Agricultural and Food Chemistry,*, **,** *53*(26), 9911–9920.

Pérez-Jiménez, J., & Saura-Calixto, F. (2015). Macromolecular antioxidants or non-extractable polyphenols in fruit and vegetables: Intake in four European countries. *Food Research International, 74*, 315–323.

Pienta, K. J., Naik, H., Akhtar, A., Yamazaki, K., Replogle, T. S., Lehr, J., Donat, T. L., Tait, L., Hogan, V., & Raz, A. (1995). Inhibition of Spontaneous Metastasis in a Rat

Prostate Cancer Model by Oral Administration of Modified Citrus Pectin. *Journal of the National Cancer Institute, 87* (5), 348–353.

Rubinstein, N., Alvarez, M., Zwirner, N. W., Toscano, M. A., Ilarregui, J. M., Bravo, A., … Rabinovich, G. A. (2004). Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell*, *5*(3), 241–251.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2015a). Pectin extracted from thermally treated olive oil by-products: Characterization, physico-chemical properties, invitro bile acid andglucose binding. *Food Hydrocolloids, 43.* 311-321

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., García, A.,& Fernández-Bolaños, J. (2015b). Novel pectin present in new olive mill wastewater with similar emulsifying and better biological properties than citrus pectin. *Food Hydrocolloids, 50*, 237-246.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A.,& Fernández-Bolaños, J. (2013). Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. *Food Research International, 54(2),* 2122–2129.

Sathisha, U. V., Jayaram, S., Harish Nayaka, M. A., & Dharmesh, S. M. (2007). Inhibition of Galectin-3 Mediated Cellular Interactions by Pectic Polysaccharides from Dietary Sources. *Glycoconjugate Journal, 24* (8), 497–507.

Seeram, N. P., Adams, L., Zhang, Y., Rupo, L., Sand, D., Scheuller, H., & Heber, D. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry extracts inhibit growth stimulate apoptosis of human cancer in vitro. *Journal of Agricultural and Food Chemistry, 54*, 9329–9339.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture, 16* (3).

Tow, W. W., Premier, R., Jing, H., & Ajlouni, S. (2011). Antioxidant and antiproliferation effects of extractable and nonextractable polyphenols isolated from apple waste

using different extraction methods. *Journal of Food Science, 76(7),* 163–172

Pectin-rich extracts from olives inhibit proliferation of Caco-2 and THP-1 cells.

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Abstract.

Three olive modified pectin extracts have been produced by heat and acid treatment of the major by-product of olive oil production. Their effect on proliferation of the colon carcinoma Caco-2 and the leukemia monocytic THP-1 cell lines has been studied in order to determine possible anti-tumor properties. All extracts inhibited proliferation at concentrations ranging from 1 to 10 mg/ml. Interestingly, none of the extracts inhibited the growth of confluent Caco-2 cells, showing the specificity of the antiproliferative effect for the transformed Caco-2 phenotype. All the extracts inhibited agglutination of red blood cells by galectin-3, a lectin involved in tumor growth, metastasis, and immune cell regulation that has been proposed as a mediator of the anti-tumor effects of modified pectins. In addition, activation of caspase-3 in THP-1 cells indicates that treatment with the pectin-rich extracts triggers apoptosis. These results point to a possible use as health-promoting food ingredients or supplements.

Keywords: Pectins, olive oil by-product, Caco-2, THP-1, galectin-3, cell proliferation.

Abbreviations: MCP modified citrus pectin.

1. Introduction.

Pectins are very complex heteropolysaccharides that are found in higher plants as structural elements of the cell wall. They are a component of soluble dietary fiber, which is poorly digested in the small intestine but ferments in the colon and plays a significant role in many physiological processes. Thus, pectin has prebiotic properties, anti-inflammatory activity, and regulates intestinal passage. In addition it aids in controlling diabetes, and in the prevention of obesity and cancer (**Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006**). Some possible applications of pectins in tissue engineering, cancer treatment, and gene delivery have been proposed (**Morris, Belshaw, Waldron, & Maxwell, 2013; Munarin, Tanzi, & Petrini, 2012**).

Modification of pectins by heat and/or chemical treatments yields lower molecular weight fragments that can be more biologically active (**Naqash, Masoodi, Rather, Wani, & Gani, 2017**). Thus, modified citrus pectins are lower molecular weight pectins that are produced by enzymatic, chemical or heat treatment of citrus pectin, yielding pectins of molecular weight up to 10 kDa. It has been found that these pectins inhibit or block aggregation of cancer cells, cellular adhesion, and metastasis (**Nangia-Makker et al., 2002**).

Although citrus fruits and apple pomace are the major sources of commercial pectin, other sources include residues from agricultural and food industries, which could lead to a revalorization of materials that are other way discarded. This is the case of alperujo, the by-product resulting from the continuous biphasic extraction of olive oil. Alperujo is a combination of the olive vegetative water and solids (skin, seeds, pulp, and pieces of stones) from the olive-pomace mill waste **(Babbar, Dejonghe, Gatti, Sforza, & Elst, 2016; Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2013**). Pectic polysaccharides rich in arabinose represent one third of the olive cell walls (**Coimbra, Cardoso, & Lopes-da-Silva, 2010**). Several million tons of alperujo are

produced every year in Spain alone, representing a disposal challenge because of its high content in organic matter and phytotoxic compounds.

We have described two procedures for thermal processing of alperujo at 50-80 ^{o}C (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, Garcia, & Fernandez-Bolanos, 2015**) or 160 ^{o}C (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2015**) that facilitates extraction of residual olive oil as well as solubilization of pectic polysaccharides and other components of interest. The resulting modified pectins extracts, which we named Pectoliv, have good physicochemical properties and health-promoting properties *in vitro*, in some cases superior to commercial pectin supplements. More recently, Pectoliv samples produced as described (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2015**), and also subjected to hydrolysis using trifluoroacetic acid, have shown to inhibit proliferation of human bladder cancer cell lines (**Bermúdez-Oria, Rodríguez-Gutiérrez, Rubio-Senent, Fernández-Bolaños, & Sánchez-Carbayo, 2018**). Now, we have used chemical treatment with citric acid or sulfuric acid to the treatment at 160 ^{o}C in order to increase the chances that the resulting modified pectins have positive biological effects. The aim of this study was to determine the effect of these Pectoliv extracts on the proliferation of two different cell lines, Caco-2 and THP-1, in order to establish whether these extracts may inhibit the growth of tumors. The effect of a commercial modified citrus pectin (MCP), Pectasol-C, was also determined for comparison. In addition, the effect of the modified pectins on agglutination of red blood cells by galectin-3 has been determined in order to assess the possible interaction of these pectins with galectin-3. It has been claimed that the beneficial health effects of modified pectins are related to binding to galectin-3, a lectin present at multiple cellular locations that has been implicated in tumorigenesis and cancer progression (**Newlaczyl & Yu, 2011**). Activation of caspase-3 and release of lactate dehydrogenase have also been determined in order to assess whether apoptosis or plain cytotoxicity are involved in any possible effect on cell proliferation.

2. Materials and methods.

2.1 Materials

Human recombinant galectin-3, neutral red, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma (Darmstadt, Germany). Alperujo, the by-product resulting from the biphasic extraction of olive oil, was provided by the oil extraction factory "Oleicola el Tejar" (Córdoba, Spain). Pectasol-C is a commercial modified citrus pectin supplement from Econugenics (Santa Rosa, CA, USA). Caco-2 and THP-1 cells were provided by the European Collection of Authenticated Cell Cultures, Public Health England. Cell culture media and serum were from Gibco, Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Pectin-rich olive extracts.

Alperujo, the main by-product of olive oil production, was steam-treated at 160 ºC for 30 min according to the procedure previously described (Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2015). The fraction released by heat treatment was ultra-filtered using a 3 kDa membrane. The resulting low molecular weight pectin extracts were precipitated in 80 % ethanol and allowed to dry (Pectoliv-1). The same procedure was also carried out in the presence of 0.5% (w/w) citric acid or sulfuric acid during steam treatment for production of Pectoliv-2 and Pectoliv-3, respectively.

2.3 Characterization of pectin extracts.

Galacturonan (anhydrogalacturonic acid) was determined according to the m-hydroxydiphenyl method as described for uronic acids (**Blumenkr & Asboehan, 1973**). Glycosyl compositions were determined by gas chromatography (GC) after conversion to alditol acetates. Individual neutral sugars were analyzed from samples with initial trifluoroacetic acid (TFA) hydrolysis (2 N TFA at 121 ºC for 1 h) prior to reduction, acetylation and analysis by GC (**Englyst & Cummings, 1984**) using inositol as internal standard. Calibration was performed using a series of standard solutions of L-rhamnose, L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose. Chromatographic

conditions were as previously described (**Lama-Munoz, Rodriguez-Gutierrez, Rubio-Senent, & Fernandez-Bolanos, 2012**).

Total phenolic content was determined using the Folin–Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents (Singleton & Rossi, 1965). Protein was determined using the micro Kjeldahl method by applying the conversion factor 6.25 (**Gibson, 1904**). Ash was determined according to the AOAC procedure (**AOAC, 1990**).

The molecular weight distribution of the purified polysaccharide-enriched extracts was estimated by high performance size exclusion chromatography (HPSEC) using two different columns (300 x 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA, USA) in sequence: TSKgel GMPWXL (dextran MW<50000KDa) and TSKgel G3000PWXL (dextran MW<60 KDa), as previously described (Dos-Santos, Jimenez-Araujo, Rodriguez-Arcos, & Fernandez-Trujillo, 2011). The system was calibrated by regression analysis using 252, 110, 70, 40, 6 kDa dextrans and glucose standards.

2.4 Cell culture and treatment.

Caco-2 and THP-1 cells were kept at 5% CO_2 in Dulbecco's Modified Eagle Medium (1000 mg/ml glucose, 110 mg/ml pyruvate, and 580 mg/ml glutamine) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 u/ml penicillin, and 100 g/ml streptomycin. Fetal bovine serum was heat-inactivated at 56 °C for 30 minutes. Caco-2 cells were subcultured once a week using trypsin-ethylenediaminetetraacetic acid, and medium was renewed once in between passages. THP-1 cells were subcultured every 2–3 days by resuspension in fresh medium.

Treatments were carried out under the same standard culture conditions. The lyophilized pectin extracts were dissolved in Hank´s Balanced Salt Solution (HBSS) at 100 mg/ml, heated at 100 ^{o}C for 30 minutes, and diluted with culture medium as required. Cells were seeded in 96 well microplates (4 x 10^4 or 14 x 10^4 cells/well, 50 □l/well). Extracts were added in the same volume (50 □l/well) to achieve a final concentration of 0.37 to 10 mg extract/ml. Caco-2 and THP-1 cells were incubated for up to 4 and 9 days,

respectively. In addition to the cell viability assay as shown below, cells were inspected under the phase contrast microscope.

2.5 Cell proliferation assays

Proliferation of adherent (Caco-2) and suspended (THP-1) cells was determined by measuring viability at different times using the neutral red and MTT assays, respectively. For the neutral red assay, cells in 96-well plates were incubated in fresh culture medium containing the vital stain neutral red (50 µg/ml) for 30 minutes. Cells were then washed using HBSS, and the stain was extracted using acetic acid (75 µl, 1% (v/v) in ethanol 50% (v/v)). Absorbance was measured at 550 nm using a plate reader (**Borenfreund & Puerner, 1985; Girón-Calle, Alaiz, & Vioque, 2010**). For the MTT assay, cells in 96-well plates were incubated in culture medium containing MTT (0.5 mg/ml) for 60 minutes. The blue formazan crystals formed by reduction of MTT were dissolved by addition of 100 µL HCl (0.1 N) in isopropanol, and absorbance at 570 nm with subtraction of background at 630 nm was measured using a plate reader (**Girón-Calle et al., 2010; Kops, West, Leach, & Miller, 1997**). Data on cell proliferation was analyzed by one way analysis of variance followed by Tukey's test for pairwise multiple comparisons (n=6).

2.6 Assays for LDH release and caspase-3 activation.

A commercial lactate dehydrogenase (LDH) cytotoxicity assay kit (Pierce-Thermo Scientific, Rockford IL, USA) was used to determine LDH release into the culture medium. The assay is based on the conversion of lactate to pyruvate catalyzed by LDH resulting in reduction of NAD^+ to NADH, which is then used by diaphorase to reduce a tetrazolium salt into a red formazan that absorbs at 490 nm. Reagents were prepared and reactions carried out as instructed in the kit. Incubations for the LDH assay were carried out in 96 well plates using the same routine culture medium as described above, but containing only 5 % FBS and no phenol red to reduce background absorbance. Absorbance was measured at 490 nm with subtraction of background at 680 nm using a plate reader. Absorption of a no reagent blank was subtracted from the samples. Two sets of replicate

incubations were carried out for each set of treatments, one for determination of LDH in the extracellular medium, and one for determination of total LDH by treatment with lysis buffer. LDH release was given as the percentage of total LDH that was released into the extracellular medium.

Caspase-3 activity was measured using the EnzChek caspase-3 assay kit (Molecular Probes, Eugene OR, USA). This kit is based on a bis amide derivative of rhodamine, rhodamine 110 bis-(N-CBZ-L-aspartyl-Lglutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD–R110), which is non-fluorescent and has two peptides blocking amino groups in the fluorophore rhodamine 110. Caspase-3 targets these peptides and releases the fluorophore, which has excitation and emission peaks at 496 and 520 nm, respectively. Cells were seeded in 12 well plates, collected, washed, and cell lysates were prepared and assayed for caspase-3 activity as instructed in the kit.

2.7 Agglutination assay

Glutaraldehyde-fixed, trypsinized rat erythrocytes were prepared as follows for agglutination assays. The pellet resulting from centrifugation of rat blood at 500 g for 10 min was washed three times by resuspension in PBS and centrifugation at 500 g for 10 minutes. The pellet was then resuspended in PBS (10% v/v) containing 0.5% (v/v) glutaraldehyde and incubated in a shaker for 1 hour at room temperature. Erythrocytes were recovered by centrifugation and washed with PBS three times as previously described, and were resuspended in PBS (20 % v/v) containing sodium azide (0.1% w/v). The fixed erythrocytes were trypsinized by incubation with trypsin (1 mg/ml erythrocytes) for 30 minutes at 37º C, and washed with PBS three times as described above (**Marquardt & Gordon, 1975**).

Agglutination assays were carried out by incubating the trypsinized, glutaraldehyde fixed rat erythrocytes (40 □L, 1.6 mg, 4 % w / v in PBS buffer) in the presence of galectin 3 (0.5 □g/well) in 96 U shape well microplates for 1 hour at room temperature. Because the commercial galectin-3 preparation included lactose as a stabilizer, it was previously dialyzed against phosphate saline buffer in order to remove

the sugar that could interfere with agglutination. Galectin-3 was added to erythrocytes as a positive control of agglutination and incubations of erythrocytes with no sample or no galectin-3 added were used as a negative control.

2.8 Statistics

The SigmaPlot program (Systat Software Inc., San Jose, California) was used for ANOVA and logistic four parameter non-linear regression analyses.

3. Results

3.1 Pectin extraction and characterization.

The chemical composition of the three Pectoliv preparations and MCP are shown in table 1, upper panel. The most important difference between Pectolivs and MCP is the content in phenolics, which was between 6 and 8 % in the former, but only 0.15 % in the latter. Content in neutral sugars and proteins was also higher in Pectolivs, while content in uronic acid was higher in MCP. The three Pectoliv preparations cannot be considered pectin preparations according to the US Pharmacopeia because they do not meet the criteria of containing at least 74% galacturonic acid. The most important differences in the composition among the three olive extracts were the contents in uronic acid and neutral sugars, which decreased from Pectoliv 1 to Pectoliv 3, while the content in ashes increased. The content in polyphenols did not change much among Pectolivs.

Analysis of the sugar composition revealed that the three Pectoliv preparations have similar compositions as compared to MCP (**table 1, lower panel**). About half of the sugar residues were uronic acid, and the percentage of xylose and galactose were between 13 and 20 %. Rhamnose, arabinose, mannose, and glucose were between 2 and 5 %, and fucose was hardly detectable. The sugar composition of MCP was clearly different, having a lot more glucuronic acid, 91 %, and lower concentrations of all other sugars.

As shown in **figure 1**, estimation of molecular weight by size exclusion chromatography indicated that the Pectoliv preparations included molecules ranging

from a few kDa up to several hundred kDa, with a peak and / or shoulder with an average size between 40 and 6 kDa. This is consistent with profiles previously described for other Pectoliv preparations that also suffered acid treatment, and with the modified pectin nature of these extracts (Bermúdez-Oria et al., 2018). MCP had a similar profile, plus an additional peak corresponding approximately to the molecular weight of single sugars.

Table1. Chemical composition (% weight, upper panel) and sugar composition (% molar composition, lower panel) of MCP and Pectolivs.

	MCP	**Pectoliv-1**	**Pectoliv-2**	**Pectoliv-3**
		% (w/w)		
Uronic acid	51.52 ± 7.78	40.94 ± 4.27	30.74 ± 1.66	24.57 ± 0.97
Neutral sugars	5.24 ± 0.07	37.03 ± 0.84	34.11 ± 1.95	23.80 ± 1.20
Phenol	0.15 ± 0.01	6.70 ± 0.38	6.33 ± 0.31	7.92 ± 0.51
Protein	3.69 ± 0.06	9.63 ± 0.13	9.31 ± 0.25	7.50 ± 0.38
Ash	20.33 ± 0.19	3.61 ± 0.50	13.81 ± 0.56	26.00 ± 1.64
Moisture	11.43 ± 0.01	6.20 ± 0.01	6.08 ± 0.01	7.70 ± 0.01
Total %	92.36	104.11	100.38	97.49
		% molar		
Rhamnose	1.69 ± 0.11	3.95 ± 0.38	5.43 ± 0.25	5.02 ± 0.40
Fucose	0.12 ± 0.03	0.05 ± 0.01	0.03 ± 0.01	traces
Arabinose	1.08 ± 0.14	6.78 ± 0.22	4.43 ± 0.14	3.21 ± 0.09
Xylose	0.49 ± 0.04	17.70 ± 0.36	19.45 ± 1.80	17.17 ± 1.19
Mannose	0.56 ± 0.07	2.40 ± 0.79	3.95 ± 0.30	3.95 ± 0.01
Galactose	4.67 ± 0.08	13.78 ± 0.79	14.69 ± 0.34	15.91 ± 1.32
Glucose	0.63 ± 0.01	2.84 ± 0.26	4.60 ± 0.2	3.95 ± 0.28
Uronic acid	90.77 ± 0.35	52.50 ± 5.51	47.42 ± 2.54	50.79 ± 2.01

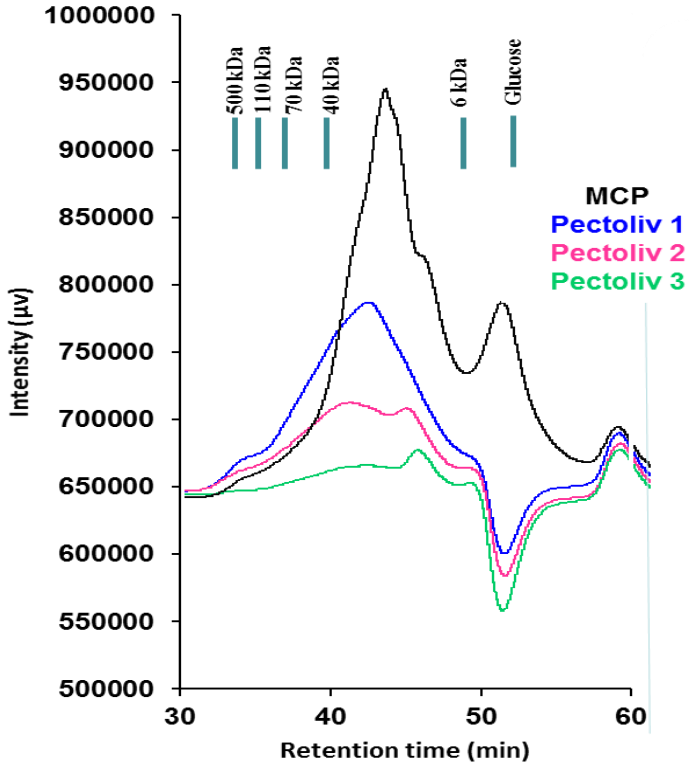

Figure 1. Molecular weight distribution of Pectoliv 1 to 3 and MCP by size exclusion chromatography. Numbers above the peaks represent molar masses of dextran standards.

3.2 Effect on cell proliferation.

Caco-2 and THP-1 cells were used as *in vitro* models to determine the effect of the pectin-rich extracts from olive in proliferation of cancerous cells as compared to the effect of MCP. Caco-2 are adherent cells that were originally isolated from a human colon carcinoma (**Engle, Goetz, & Alpers, 1998**), and THP-1 cells grow in suspension and were isolated from a monocytic leukaemia patient (**Tsuchiya et al., 1980**). Caco-2 cells were exposed to increasing concentrations of the extracts and MCP for up to 7 days, and cell proliferation was determined by measuring uptake of the vital stain neutral red at the

end of the different incubation periods. These treatments were carried out in complete culture medium by addition of the samples at the same time the cells were seeded. A concentration-dependent inhibition of cell proliferation was caused by MCP and Pectolivs as shown in **figure 2**. While the lowest concentration, 0.37 mg/ml, did not inhibit proliferation at all, the highest concentration, 10 mg/ml, completely inhibited proliferation. Pectolivs at 3.33 mg/ml inhibited proliferation completely, but the same concentration of MCP still allowed some proliferation. Interestingly, treatment for 7 days with 1.11 mg/ml of any of the modified pectins actually enhanced cell proliferation.

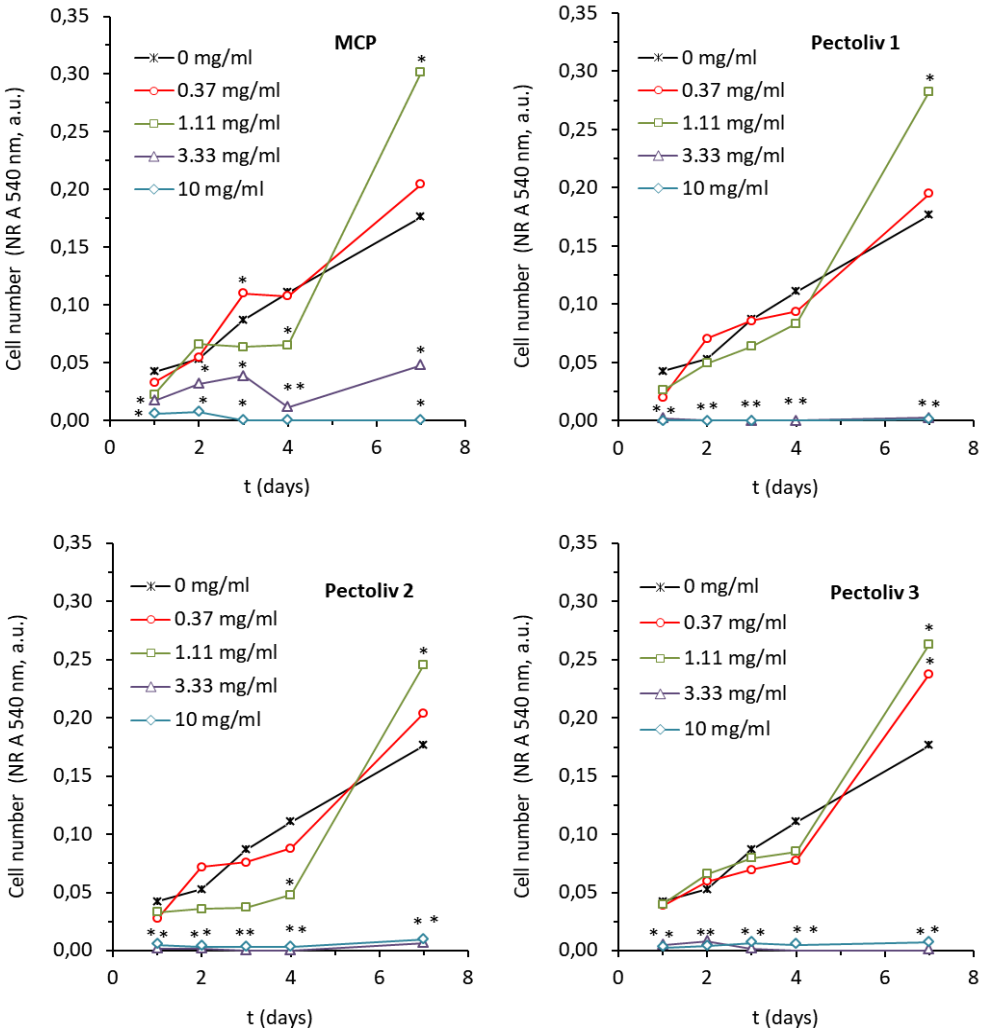

Figure 2. Effect of Pectolivs and MCP on proliferation of Caco-2 cells. Cells ($4x10^3$ cells/well) were seeded in the presence of increasing concentrations of Pectolivs and MCP and allowed to proliferate for one to seven days. Cell number was then estimated by determination of neutral red uptake. Data represent average of six replicates, error bars are not shown for clarity. Asterisks indicate statistically significant difference between treatment and vehicle control for each incubation period (one way ANOVA-Tukey test, $p<0.05$).

THP-1 cells were exposed to the same concentrations of MCP and Pectolivs than Caco-2 cells, but because these cells grow faster, exposure was carried out for up to four days instead of seven (**figure 3**). Proliferation was inhibited by MCP and Pectolivs even at the lowest concentration. A comparison of the doses that caused inhibition of proliferation by half (ED50) after incubation for three days is shown in table 2. These data highlights the fact that Caco-2 cells were more susceptible to inhibition of proliferation than THP-1 cells, and that the effect of Pectoliv was higher than that of MCP.

As mentioned before, incubation of Caco-2 cells with Pectolivs and MCP at low concentration for seven days reversed the inhibition seen after shorter incubation times, and actually enhanced proliferation (**figure 2**). This might be related with the fact that Caco-2 cells differentiate into an enterocyte-like phenotype when they are allowed to grow as confluent monolayers (**Engle et al., 1998**). In order to test this hypothesis, Caco-2 cells were seeded at a higher density and allowed to grow for two days before exposure to the models pectins. This time frame allows for the cells to be exposed while they are differentiating but still growing, although at a lower rate than the same cells in non-confluent conditions. This experiment revealed that the differentiating Caco-2 cells were mostly immune to the inhibitory effect of the modified pectins **(figure 4**). Although there were some statistically significant effects on cell proliferation, these were lower than 10 % at the best and do not follow any concentration dependent pattern.

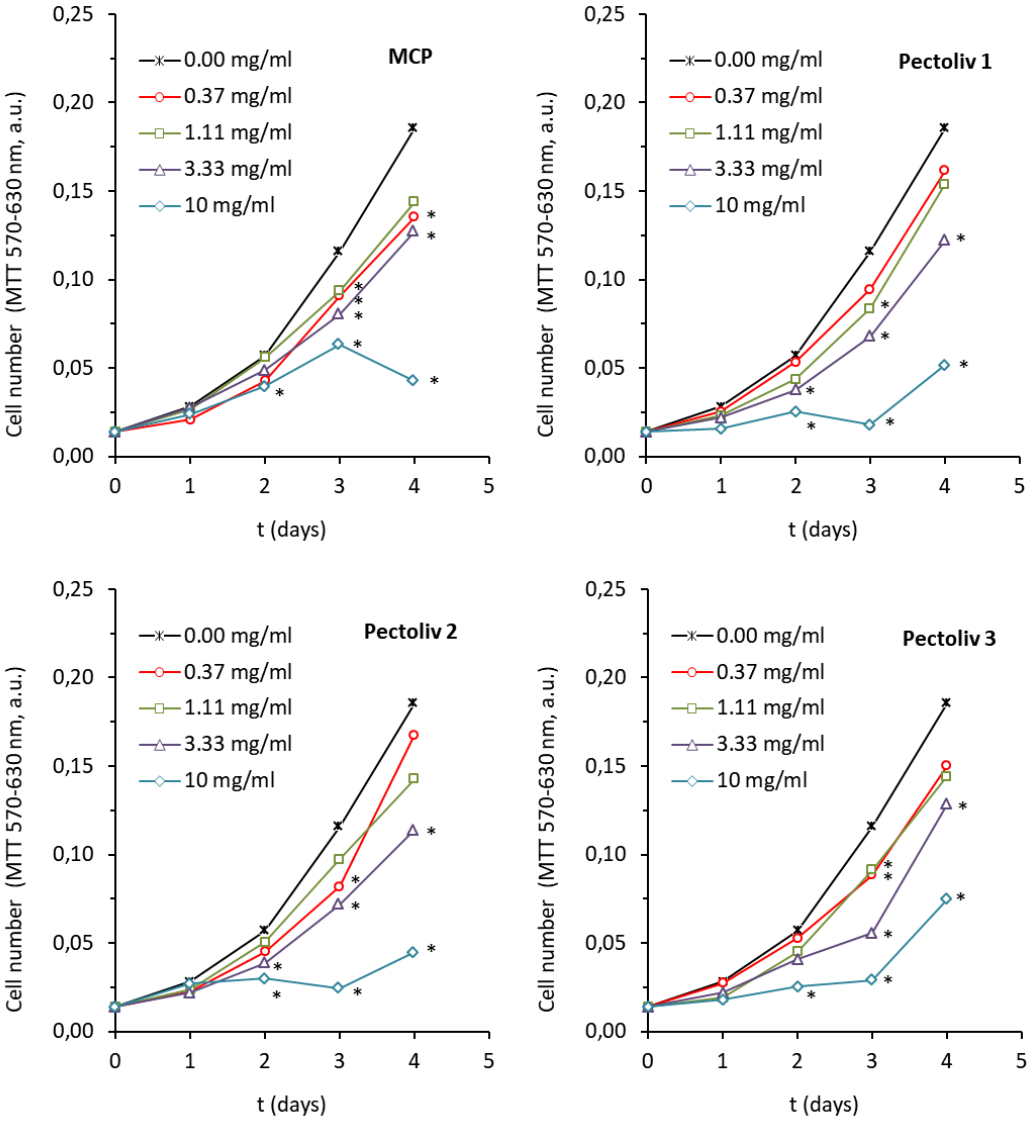

Figure 3. Effect of Pectolivs and MCP on proliferation of THP-1 cells. Cells ($4x10^3$ cells/well) were seeded in the presence of increasing concentrations of Pectolivs and MCP and allowed to proliferate for up to four days. Cell number was then estimated by measuring reduction of MTT. Data represent average of six replicates, error bars are not shown for clarity. Data represent average of six replicates, error bars are not shown for clarity. Asterisks indicate statistical significant difference between treatment and vehicle control for each incubation period (one way ANOVA-Tukey test, $p<0.05$).

Table 2. Inhibition of cell proliferation and inhibition of erythrocyte agglutination by galectin-3 in the presence of the modified pectins. ED50 (mg/ml) for inhibition of cell proliferation after incubation for three days was calculated using logistic four parameter non-linear regression of some of the data plotted in **figures 2** (Caco-2 cells) and 4 (THP-1 cells). Inhibition of agglutination is expressed as the minimum concentration of modified pectins (mg/ml) inhibiting agglutination of red blood cells by galectin-3.

	MCP	**Pectoliv-1**	**Pectoliv-2**	**Pectoliv-3**	**camptothecin**
ED50 Caco-2	2.56	1.31	1.96	2.01	0.04
ED50 confluent Caco-2	-	-	-	-	0.23
ED50 THP-1	> 10	3.89	4.67	3.75	0.01
lowest concentration inhibiting agglutination	32	8	8	4	

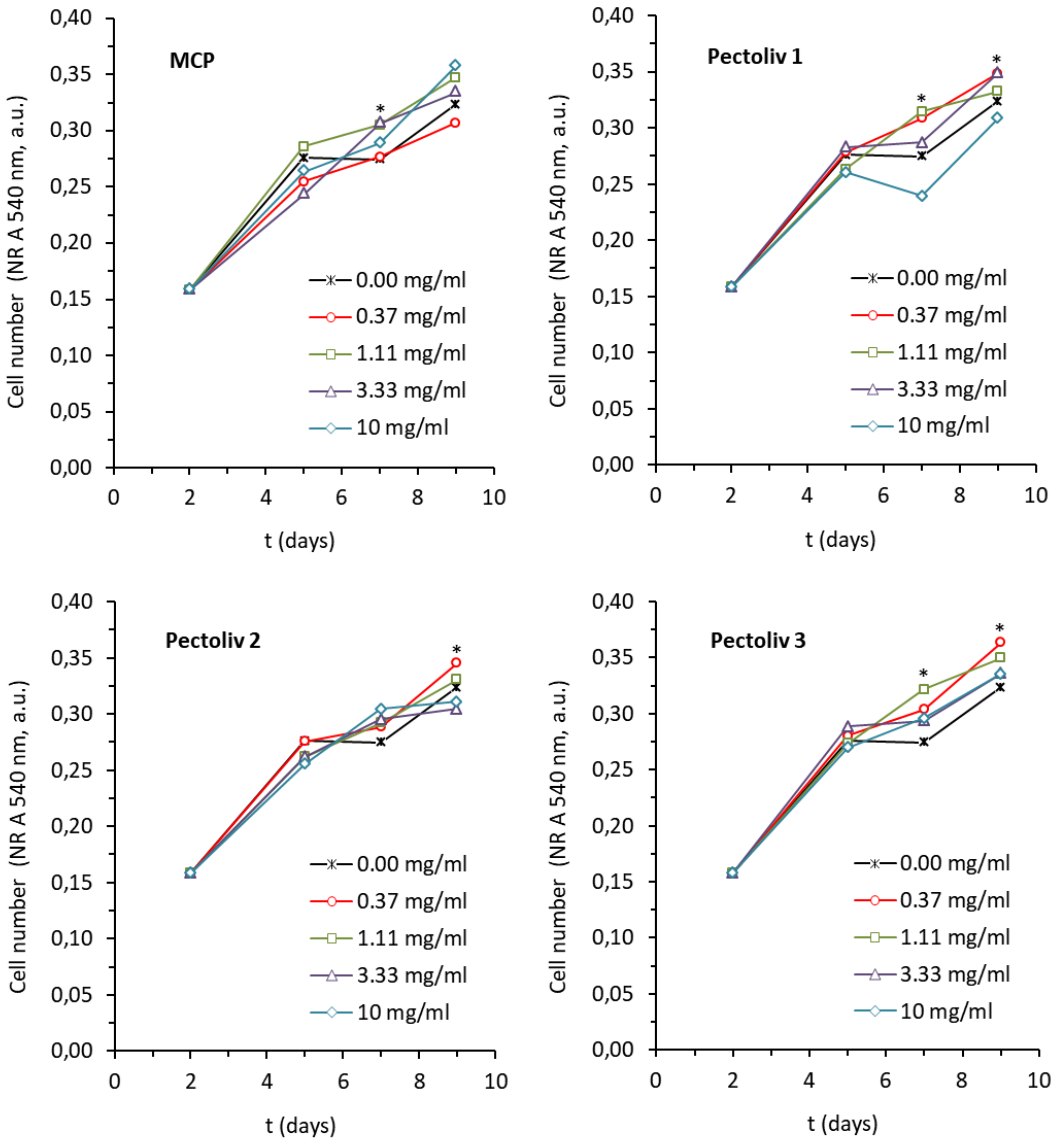

Figure 4. Effect of Pectolivs and MCP on proliferation of confluent Caco-2 cells. Cells ($14x10^3$ cells/well) were seeded and incubated for two days. Increasing concentration of Pectolivs and CMP were then added and cells were incubated for up to seven more days. Cell number was estimated by determination of neutral red uptake. Data represent average of six replicates, error bars are not shown for clarity. Data represent average of six replicates, error bars are not shown for clarity. Asterisks indicate statistically significant difference between treatment and vehicle control for each incubation period (one way ANOVA-Tukey test, $p<0.05$).

3.3 Activation of caspase-3 and LDH release.

Inhibition of cell proliferation can be due to an actual slow-down of cell growth, or to induction of cell death via apoptosis and/or necrosis. Caspase-3 is one of the effector caspases that are activated by initiator proteases of the same family, which mediate both the intrinsic and extrinsic pathways for apoptosis (**Elmore, 2007**). Release of the cytosolic enzyme lactate dehydrogenase (LDH) to the extracellular medium is a hallmark of cell membrane rupture and is used as a cytotoxicity assay. While apoptosis is a programmed chain of events that results in cell death, cellular death by necrosis is characterized by random cell disruptions that cause release of cellular contents without mediation of the caspase pathway. Activation of caspase-3 and LDH release were determined after incubation of THP-1 cells with MCP and Pectolivs in order to gain insight on whether these treatments caused programmed cell death and/or necrosis.

As shown in **figure 5**, treatment with any of the Pectolivs at a concentration of 10 mg/ml for 48 hours caused activation of caspase-3, while treatment with MCP did not. The activation of caspase-3 by Pectolivs somehow resembles the proliferation curves shown in **figure 3**, indicating that apoptosis might be involved in the antiproliferative effect of these samples. LDH release into the media after incubation with Pectolivs or MCP was lower than 10 % in all cases and no significant difference was found as compared to control (not shown).

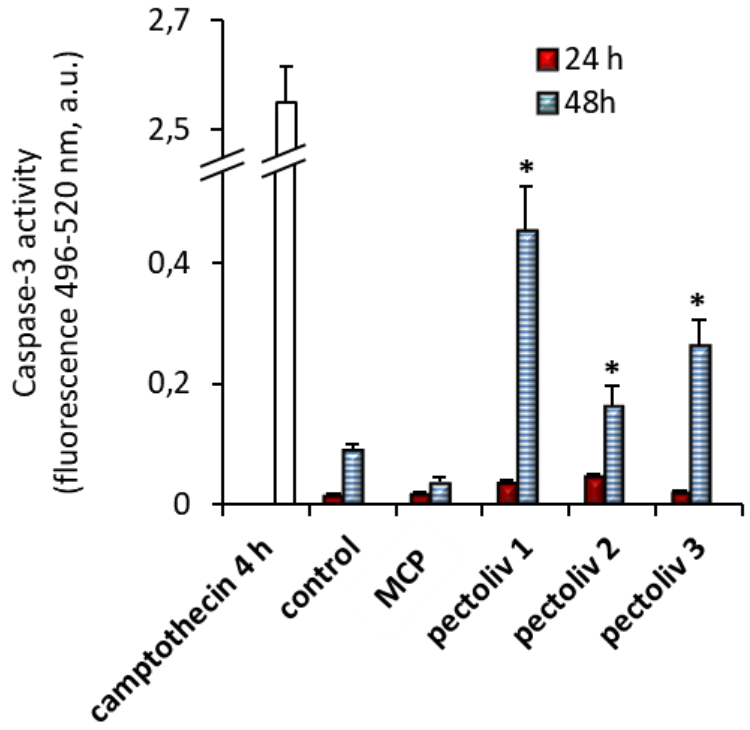

Figure 5. Caspase-3 activation in cells treated with Pectolivs and MCP. THP-1 cells ($4x10^3$ cells/well) were seeded in the presence of the modified pectins (10 mg/ml) and incubated for 24 and 48 hours. As a positive control, cells were treated with camptothecin for 4 hours. Data represent average of five replicates, error bars represent standard deviation. Asterisks indicate statistically significant differences between treatments and vehicle control (one way ANOVA-Tukey test, $p<0.05$).

3.4 Effect on agglutination of red blood cells by galectin-3

The possible disruption of the agglutination of red blood cells by galectin-3 was used to assess the possible interaction of this protein with MCP and Pectolivs. Concentrations between 4 and 60 mg/ml were tested to determine inhibition of the agglutination caused by 0.5 mg/well galectin-3. Both Pectolivs and MCP were able to inhibit the agglutination of red blood cells by galectin-3, although Pectolivs proved to be more effective than MCP, as shown in table 2.

4. Discussion

Our group has developed procedures for thermal treatment of the alperujo by-product at low (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, Garcia, et al., 2015**) and high (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2015**) temperature so that both extraction of the residual oil and release of bioactive components are facilitated. Fractions resulting from the high-temperature treatment have been called Pectolivs in general because of their relatively high content in pectin, although they do not meet the USP criteria for pectins of having a content in galacturonic acid of at least 74%. The Pectoliv-1 preparation that has been used in the present work has been produced by high temperature treatment of alperujo as described (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2015**), while Pectoliv-2 and Pectoliv-3 represent modifications of the original procedure to include treatment with citric and sulphuric acid, respectively. Steam treatment promoted the cleavage of the galacturonic backbone and arabinogalactan sidechains, as well as other polysaccharides differing in their sugar composition that are probably derived from arabinoxylan, glucomannan and xyloglucan, which were previously described as constituents of olive pulp (**Jimenez, Guillen, Fernandezbolanos, & Heredia, 1994**). The acid treatment at high temperature did affect the uronic acid content and reduced the molecular weight, as might be expected by acid hydrolysis (**Diaz, Anthon, & Barrett, 2007**).

All Pectoliv preparations have a complex chemical composition characterized by the formation of complexes of proteins, polyphenols and pectins at high temperatures. The composition of Pectoliv reflects the fact that olive oil extraction and subsequent thermal treatment of the alperujo by-product leads to formation of complexes that link pectins, proteins and phenols together (**Bermúdez-Oria et al., 2018; Capasso, De Martino, & Arienzo, 2002**). Differences in the composition of the olives and the resulting by-product of olive oil extraction, as well as modifications in the thermal treatment, often result in variability in the composition of different Pectoliv preparations. This is especially

true for the content in phenols that can vary from 4 up to 59% (w/w). Pectolivs in this paper are characterized by a quite homogeneous composition with a relatively low content in phenols, 6-8 %, as compared to other Pectolivs (**Bermúdez-Oria et al., 2018; Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2015**).

Caco-2 cells have been used as a model of exposure to components in the diet and drugs (**Giron-Calle et al., 2004; Sánchez-Vioque et al., 2016**), and as a model of absorption in the gut when they are allowed to differentiate in vitro to an enterocyte-like phenotype (**Artursson, Palm, & Luthman, 2001; Girón-Calle et al., 2010**). When Caco-2 cells are allowed to grow in confluent cultures they differentiate into polarized cells that resemble the healthy enterocyte in morphology and physiology (**Engle et al., 1998**). Thus, the same cell line can be used to model both cancerous, transformed cells, and healthy epithelial cells (**Giron-Calle et al., 2004**). Results of exposure of Caco-2 cells to Pectolivs and MCP reveal that inhibition of proliferation is dependent on the transformed Caco-2 phenotype, supporting the view that this phenomenon is not due to non-specific toxicity to cells. Because we are dealing with an *in vitro* model of tumors in the colon, the antiproliferative activity of Pectolivs would not depend on the absorption, or lack of, from the gut into the bloodstream.

THP-1 cells complement very well Caco-2 cells as an *in vitro* model of exposure of tumors to bioactive components. This is so because they are cells of monocytic nature, thus belonging to the immune system, and grow in suspension. Exposure of THP-1 cells to MCP and Pectolivs also revealed a marked inhibition of proliferation. The antiproliferative activity of Pectolivs on both Caco-2 and THP-1 cells was higher than the activity of MCP. Previous reports show that pectins, including MCP, apple pectins, and pectin oligosaccharides inhibit proliferation by inducing apoptosis (**Jackson et al., 2007; Leclere et al., 2015; Li et al., 2012**), reviewed in (**Leclere, Van Cutsem, & Michiels, 2013**). Activation of caspase-3 as shown here clearly shows that induction of apoptosis may as well be involved in the antiproliferative effects of Pectolivs. Surprisingly, we did not find activation of caspase-3 by MCP as compared to the control, but this might be due to the fact that MCP has a lower inhibitory activity than Pectolivs, which is just starting to show

after two days of treatment. The lack of release of LDH upon treatment with Pectolivs further support the view that apoptosis, and not non-specific cellular toxicity, is involved in the inhibition of cells proliferation.

Several reports found that interaction with galectin-3 appears to be involved in the antitumor effects of pectins, including MCP. Our own group recently found a decreased expression of galectin-3 in bladder cancer cells lines treated with Pectolivs, and inhibition of galectin-3 agglutination of red blood cells by the same Pectoliv preparations (**Bermúdez-Oria et al., 2018**). Our data is consistent with these results, showing that antiproliferative activity is paralleled by inhibition of agglutination by galectin-3. Interestingly, expression of galectin-3 correlates with transformation and invasiveness in colon cancer as determined in a large number of primary and metastatic tumors (**Schoeppner, Raz, Ho, & Bresalier, 1995**) and (**Nakamura et al., 1999**). Other report described an initial decrease in expression of galectin-3, followed by an increase in expression, during progression of colon cancer (**Sanjuan et al., 1997**). Considering these reports, it is tempting to propose that expression of galectin-3 decreases upon differentiation of Caco-2 cells, and that this might explain why proliferation of confluent Caco-2 cells is no longer inhibited by the modified pectins. Nevertheless, galectin-3 expression in the plasma membrane of Caco-2 cells increased between four and eight times with differentiation, as determined using three different proteomic approaches to study changes in protein expression during differentiation of these cells (**Pshezhetsky et al., 2007**), and it has actually been reported to be necessary for differentiation of an epithelial cell line (**Hikita et al., 2000**). Galectin-3 expression in Caco-2 cells may also be increased by exposure to xenobiotics (**Isoda, Talorete, Han, & Nakamura, 2006**). It should be kept in mind that galectin-3 can be found in different intracellular locations and carries out different functions, so that simply considering expression levels may fail to indicate whether this protein is involved or not in a phenomenon.

Galectin-3 is also involved in regulation of the immune system, and it is especially expressed in monocytes and macrophages, including the THP-1 cell line. In these cells, it is upregulated upon stimulation with lipopolysaccharide (**Dabelic, Novak, Goreta, &**

Dumic, 2012). Galectin-3 is involved in cancers of the blood cells, especially leukemia (**Ruvolo, 2016**). The THP-1 cell line is one example of how cells of the immune system can express galectin-3 in different locations, and can at the same time bind and respond to this protein when present in the extracellular medium, modulating immune response and progression and metastasis of blood cell cancers (**Dabelic et al., 2012**).

In conclusion, our data clearly shows that the three Pectoliv preparations used for this study inhibit proliferation of two *in vitro* models of cancerous cells. One is a model of tumors in the colon, and the other is a model of leukemia. The inhibitory effect was higher than the effect of MCP that was used as reference material. In parallel with this activity, the three Pectoliv preparations induced apoptosis as determined by activation of caspase-3, and inhibited agglutination of red blood cells by galectin-3.

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6. References.

AOAC (Official Methods of Analysis) (1990). (15th ed.). Food composition, vol. 1 (pp. 915–919) Arlington, Virginia: AOAC International.

Artursson, P., Palm, K., & Luthman, K. (2001). Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv Drug Deliv Rev,* 46(1-3), 27-43.

Babbar, N., Dejonghe, W., Gatti, M., Sforza, S., & Elst, K. (2016). Pectic oligosaccharides from agricultural by-products: production, characterization and health benefits. *Critical Reviews in Biotechnology,* 36(4), 594-606.

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Fernández-Bolaños, J., & Sánchez-Carbayo, M. (2018). Olive extract rich in polysaccharides with antioxidant and antiproliferative activity on bladder cancer cells. *Manuscript submitted for publication*.

Blumenkr, N., & Asboehan, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry,* 54(2), 484-489.

Borenfreund, E., & Puerner, J. A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol Lett,* 24(2-3), 119-124.

Capasso, R., De Martino, A., & Arienzo, M. (2002). Recovery and characterization of the metal polymeric organic fraction (polymerin) from olive oil mill wastewaters. *J Agric Food Chem,* 50(10), 2846-2855.

Coimbra, M. A., Cardoso, S. M., & Lopes-da-Silva, J. A. (2010). Olive pomace, a source for valuable arabinan-rich pectic polysaccharides. *Top Curr Chem,* 294, 129-141.

Dabelic, S., Novak, R., Goreta, S. S., & Dumic, J. (2012). Galectin-3 expression in response to LPS, immunomodulatory drugs and exogenously added galectin-3 in monocyte-like THP-1 cells. *In Vitro Cell Dev Biol Anim,* 48(8), 518-527.

Diaz, J. V., Anthon, G. E., & Barrett, D. M. (2007). Nonenzymatic degradation of citrus pectin and pectate during prolonged heating: effects of pH, temperature, and degree of methyl esterification. *J Agric Food Chem,* 55(13), 5131-5136.

Dos-Santos, N., Jimenez-Araujo, A., Rodriguez-Arcos, R., & Fernandez-Trujillo, J. P. (2011). Cell Wall Polysaccharides of Near-Isogenic Lines of Melon (Cucumis melo L.) and

Their Inbred Parentals Which Show Differential Flesh Firmness or Physiological Behavior. *Journal of Agricultural and Food Chemistry,* 59(14), 7773-7784.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology,* 35(4), 495-516.

Engle, M. J., Goetz, G. S., & Alpers, D. H. (1998). Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. *J Cell Physiol,* 174(3), 362-369.

Englyst, H. N., & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst,* 109(7), 937-942.

Gibson, R. B. (1904). The determination of nitrogen by the Kjeldahl method. *J Am Chem Soc,* 26(1), 105-110.

Girón-Calle, J., Alaiz, M., & Vioque, J. (2010). Effect of chickpea protein hydrolysates on cell proliferation and in vitro bioavailability. *Food Research International,* 43(5), 1365-1370.

Giron-Calle, J., Vioque, J., del Mar Yust, M., Pedroche, J., Alaiz, M., & Millan, F. (2004). Effect of chickpea aqueous extracts, organic extracts, and protein concentrates on cell proliferation. *J Med Food,* 7(2), 122-129.

Hikita, C., Vijayakumar, S., Takito, J., Erdjument-Bromage, H., Tempst, P., & Al-Awqati, Q. (2000). Induction of Terminal Differentiation in Epithelial Cells Requires Polymerization of Hensin by Galectin 3. *The Journal of Cell Biology,* 151(6), 1235-1246.

Isoda, H., Talorete, T. P. N., Han, J., & Nakamura, K. (2006). Expressions of galectin-3, glutathione S-transferase A2 and peroxiredoxin-1 by nonylphenol-incubated Caco-2 cells and reduction in transepithelial electrical resistance by nonylphenol. *Toxicology in Vitro,* 20(1), 63-70.

Jackson, C. L., Dreaden, T. M., Theobald, L. K., Tran, N. M., Beal, T. L., Eid, M., . . . Mohnen, D. (2007). Pectin induces apoptosis in human prostate cancer cells: correlation of apoptotic function with pectin structure. *Glycobiology,* 17(8), 805-819.

Jimenez, A., Guillen, R., Fernandezbolanos, J., & Heredia, A. (1994). Cell-wall composition of olives. *Journal of Food Science,* 59(6), 1192-&.

Kops, S. K., West, A. B., Leach, J., & Miller, R. H. (1997). Partially purified soy hydrolysates retard proliferation and inhibit bacterial translocation in cultured C2BBe cells. *J Nutr,* 127(9), 1744-1751.

Lama-Munoz, A., Rodriguez-Gutierrez, G., Rubio-Senent, F., & Fernandez-Bolanos, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids,* 28(1), 92-104.

Leclere, L., Fransolet, M., Cote, F., Cambier, P., Arnould, T., Van Cutsem, P., & Michiels, C. (2015). Heat-Modified Citrus Pectin Induces Apoptosis-Like Cell Death and Autophagy in HepG2 and A549 Cancer Cells. *PLoS One,* 10(3).

Leclere, L., Van Cutsem, P., & Michiels, C. (2013). Anti-cancer activities of pH- or heat-modified pectin. *Frontiers in Pharmacology,* 4, 8.

Li, Y. H., Liu, L., Niu, Y. B., Feng, J., Sun, Y., Kong, X. H., . . . Mei, Q. B. (2012). Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitis-associated colon cancer: role of galectin-3 and apoptosis in cancer prevention. *European Journal of Nutrition,* 51(1), 107-117.

Markov, P. A., Popov, S. V., Nikitina, I. R., Ovodova, R. G., & Ovodov, Y. S. (2011). Anti-inflammatory activity of pectins and their galacturonan backbone. *Russian Journal of Bioorganic Chemistry,* 37(7), 817-821.

Marquardt, M. D., & Gordon, J. A. (1975). Glutaraldehyde fixation and mechanism of erythrocyte agglutination by concanavalin A and soybean agglutinin. *Exp Cell Res,* 91(2), 310-316.

Morris, V. J., Belshaw, N. J., Waldron, K. W., & Maxwell, E. G. (2013). The bioactivity of modified pectin fragments. *Bioactive Carbohydrates and Dietary Fibre,* 1(1), 21-37.

Munarin, F., Tanzi, M. C., & Petrini, P. (2012). Advances in biomedical applications of pectin gels. *International Journal of Biological Macromolecules,* 51(4), 681-689.

Nakamura, M., Inufusa, H., Adachi, T., Aga, M., Kurimoto, M., Nakatani, Y., . . . Yasutomi, M. (1999). Involvement of galectin-3 expression in colorectal cancer progression and metastasis. *Int J Oncol,* 15(1), 143-148.

Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., & Raz, A. (2002). Inhibition of human cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin. *Jnci-Journal of the National Cancer Institute,* 94(24), 1854-1862.

Naqash, F., Masoodi, F. A., Rather, S. A., Wani, S. M., & Gani, A. (2017). Emerging concepts in the nutraceutical and functional properties of pectin—A Review. *Carbohydrate Polymers,* 168, 227-239.

Newlaczyl, A. U., & Yu, L.-G. (2011). Galectin-3 – A jack-of-all-trades in cancer. *Cancer Letters,* 313(2), 123-128.

Pshezhetsky, A. V., Fedjaev, M., Ashmarina, L., Mazur, A., Budman, L., Sinnett, D., . . . Levy, E. (2007). Subcellular proteomics of cell differentiation: Quantitative analysis of the plasma membrane proteome of Caco-2 cells. *Proteomics,* 7(13), 2201-2215.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science & Technology,* 17(1), 3-15.

Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Munoz, A., & Fernandez-Bolanos, J. (2013). Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. *Food Research International,* 54(2), 2122-2129.

Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Munoz, A., & Fernandez-Bolanos, J. (2015). Pectin extracted from thermally treated olive oil by-products: Characterization, physico-chemical properties, in vitro bile acid and glucose binding. *Food Hydrocolloids,* 43, 311-321.

Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Munoz, A., Garcia, A., & Fernandez-Bolanos, J. (2015). Novel pectin present in new olive mill wastewater with similar

emulsifying and better biological properties than citrus pectin. *Food Hydrocolloids,* 50, 237-246.

Ruvolo, P. P. (2016). Galectin 3 as a guardian of the tumor microenvironment. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1863(3), 427-437.

Sánchez-Vioque, R., Santana-Méridas, O., Polissiou, M., Vioque, J., Astraka, K., Alaiz, M., . . . Girón-Calle, J. (2016). Polyphenol composition and in vitro antiproliferative effect of corm, tepal and leaf from Crocus sativus L. on human colon adenocarcinoma cells (Caco-2). *Journal of Functional Foods,* 24, 18-25.

Sanjuan, X., Fernandez, P. L., Castells, A., Castronovo, V., van den Brule, F., Liu, F. T., . . . Campo, E. (1997). Differential expression of galectin 3 and galectin 1 in colorectal cancer progression. *Gastroenterology,* 113(6), 1906-1915.

Schoeppner, H. L., Raz, A., Ho, S. B., & Bresalier, R. S. (1995). Expression of an endogenous galactose-binding lectin correlates with neoplastic progression in the colon. *Cancer,* 75(12), 2818-2826.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture,* 16(3), 144-158.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., & Tada, K. (1980). Establishment and Characterization of a Human Acute Monocytic Leukemia-Cell Line (Thp-1). *International Journal of Cancer,* 26(2), 171-176.

Polyphenols associated to pectic polysaccharides account for most of the antiproliferative and antioxidant activities in olive extracts.

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ABSTRACT

Extracts rich in pectic polysaccharides associated to polyphenols were obtained from alperujo, the by-product of olive oil extraction, by different thermal treatment and subsequent chemical treatments. The resulting “Pectoliv” extracts exhibited strong antioxidant activity and a high antiproliferative capacity in vitro against colon carcinoma Caco-2 and leukemia monocytic THP-1 cell lines. Bleaching of the extracts with sodium chlorite removed the phenolic compounds and significantly reduced antiproliferative and antioxidant activities, confirming that these were due mainly to the associated polyphenols present in Pectoliv. However, even after bleaching, these activities remained very similar to that of a commercial citrus modified pectin obtained by chemical treatment from citrus pectin, which has been implicated in cancer treatment and prevention. Thus Pectolivs have a high potential as antiproliferative or antioxidant agents.

Keywords: cancer; alperujo; modified citrus pectin; antiproliferative activity; antioxidant activity.

1. Introduction

Alperujo is one of the most important by-products generated by the olive oil manufacturing process using the continuous two-phase extraction system, with over 4 million tonnes generated annually in Spain. Alperujo is a combination of the olive vegetative water and solids (skin, seeds, pulp, and stones) from olive-pomace mill waste (**Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2013**). Alperujo is a source of substances of high added value, including phenols, carbohydrates and proteins. The thermal treatment of alperujo (**Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz & Fernández-Bolaños, 2012**), which allows an easy separation of the solid and liquid phases, also results in the solubilization of compounds of high added value in the liquid phase. For example, considerable amounts of pectic polysaccharides can be obtained from the aqueous fraction, because one third of the olive-pulp cell wall is made up of arabinose-rich pectin polysaccharides (**Cardoso, Coimbra, & Lopes da Silva, 2003**).

Pectins are a complex polysaccharide found in the cell wall of higher plants. They contribute to soluble dietary fiber, which is incompletely processed by human digestive enzymes and not absorbable in the small intestine, but is fermented by colon bacteria (**Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006**). Moreover, it is associated with gastrointestinal health and recently there is evidence of the prevention and treatment of cancer (**Wollowski, Rechkemmer, & Pool-Zobel, 2001; Mudgil & Barak, 2013**).

Pectin treated with pH adjustment – by alkali or acid, heat or enzyme, and known as “modified pectin” – has been broken into small fragments that, in theory, with a molecular weight around 10 kDa, can be absorbed and pass into the blood circulation (**Zhang, Gao, Shi & Zhang, 2007**). Several studies proved that modified pectin has an anticancer effect, causing the reduction of cell proliferation, migration and adhesion, as well as triggering the induction of apoptosis and anti-metastasis (**Nangia-Makker et al., 2002; Liu, Huang, Yang, Lu, & Yu, 2008; Maxwell, Belshaw, Waldron, & Morris, 2012; Zhang, Xu, & Zhang, 2015; Maxwell et al., 2016**). Generally, it is thought that modified pectin is biologically active due to its galactan side chains that can bind to the pro-

metastasic protein Galectin-3 (**Maxwell et al., 2012**). Galectin-3 is a target protein that when overexpressed promotes metastasis and protects cancer cells from apoptosis (**Nangia-Makker et al., 2002**), and when its activity is blocked, inhibits cell adhesion (**Maxwell et al., 2012**) and enhances the induction of apoptosis (**Liu et al., 2008**).

Citrus fruits and apple pomace are the major sources for commercial pectin, although other sources are being introduced. Alternative sources include residues from agricultural and food industries, which could lead to a revalorization of waste materials that are otherwise discarded, as is the case with alperujo. Previous studies carried out by our group indicate that the pectic material obtained from alperujo by hydrothermal treatment (160º C/30 min) (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, & Fernandez-Bolaños, 2015a**) or by gentle heat treatment (50–80º C) (**Rubio-Senent, Rodriguez-Gutierrez, Lama-Munoz, Garcia, & Fernandez-Bolaños, 2015b**) showed different physical characteristics and biological properties. In both cases, it had a high oil capacity and emulsifying activity, and a higher capacity for binding bile acids and glucose than commercial citrus pectin. A high antiproliferative activity was observed in different bladder cancer lines from alperujo-derived pectin extracts rich in polyphenols compared with a modified citrus pectin (MCP) (Pectasol-C) or cisplatin (CDDP), a known anticancer agent (**Bermúdez-Oria et al., 2019a**). In addition, other alperujo-derived pectin extracts containing phenols inhibited proliferation of Caco-2 and THP-1 cancer cells, and also inhibited hemagglutination by galectin-3. These inhibitory effects were even higher than those of MCP (**Bermúdez-Oria et al., 2019b**).

The first objective of the present work was to investigate the effect of different chemical or thermal treatments on the composition, antioxidant effect, and antiproliferative activity on Caco-2 cells of pectic extracts obtained from alperujo. The second objective was to determine whether polyphenols are related with these activities. For this, polyphenols were removed from some of the extracts by bleaching using chlorite before assessing antioxidant capacity and the effect on proliferation of Caco-2 and THP-1 cells.

2. Material and Methods

2.1 Materials

Neutral red and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma. Pectasol-C is a commercial modified citrus pectin supplement from Econugenics (Santa Rosa, California). Caco-2 and THP-1 cells were provided by the European Collection of Authenticated Cell Cultures, Public Health England. Cell culture media and serum were from Gibco, Thermo Fisher Scientific.

2.2 Raw material

Olive pomace, or "alperujo" (a semi-solid residue composed of olive peel, pulp, seeds, and ground stones), was collected directly after the two-phase centrifugal system used in a local pomace processing mill (Marchena, Sevilla, Spain) for the extraction of olive oil.

2.3 Isolation and purification of extracts from alperujo

The hydrothermal treatment of alperujo (**Fernandez-Bolaños et al., 2010, Patent no. ES2374675**) was performed using a steam treatment reactor prototype, designed by our research group at the Instituto de la Grasa (Seville, Spain). Fresh alperujo samples (8 Kg) were treated with saturated steam for 60 min at a temperature of 80º C or 120º C, or for 30 min at a temperature of 160º C. Heating of the alperujo was performed by direct steam injection, enhancing the contact between the steam and the alperujo. After closing the steam inlet valve, the pressure was reduced to atmospheric pressure at a controlled rate. The wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain) to separate the solid and liquid phases, with subsequent ultrafiltration of the liquid phase at 3000 Da. The liquid phase higher than 3000 Da was concentrated to 2 L in a rotary evaporator, and precipitated with 70% EtOH. The alcohol-insoluble residue was freeze-dried and named according to the temperature at which it was obtained: Pectoliv-80, Pectoliv-120 and Pectoliv-160. Part of the Pectoliv-80 and -120 extracts were divided and a basic hydrolysis carried out with 1 M NaOH for 1 hour, with subsequent neutralization

with 1 M HCl. The liquid was lyophilized, and the extract were named Pectolive-80A and -120A (Fig. 1).

2.4 Bleaching of Pectoliv

Pectoliv extracts were treated with acid followed by bleaching according to Renard & Thibault (1996) with some modifications. A bleaching solution was prepared by mixing acetic acid (CH_3COOH 0.2% w/v) and sodium chlorite ($NaClO_2$ 0.2% w/v). Briefly, MCP and Pectoliv-80, -80A and -160 (7.5 % w/v) were added to the bleaching solution. The mixture was heated at 70º C for 1 hour. These steps were repeated twice. The samples were precipitated with 70% EtOH, filtered and rinsed with EtOH. The bleached phenolic residues were removed by washing and ultrafiltration at 1000 Da, and then lyophilized. Bleached extracts were named Pectoliv-80B, -80AB, -160B and MCP-B (Fig. 1).

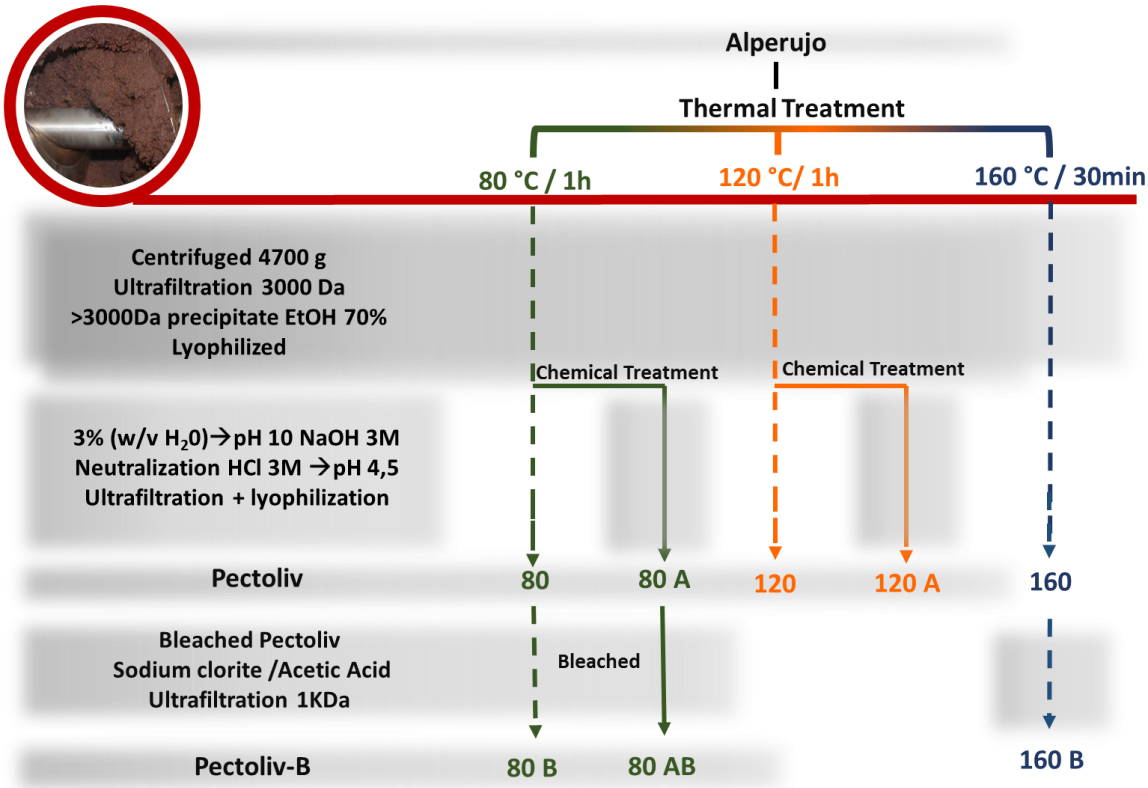

Figure 1. Scheme of the extraction procedure employed to obtain the five different extracts, named Pectoliv, from olive by-product by different thermal and chemical treatments. Three of the extracts were bleached with sodium chlorite in acidic conditions.

2.5 Characterization of pectin extracts

Galacturonan (anhydrogalacturonic acid) was determined according to the m-hydroxydiphenyl method described by **Blumenkrantz & Asboe-Hansen** (1973) for uronic acids.

Glycosyl compositions were determined by gas chromatography (GC) after conversion to alditol acetates. Individual neutral sugars were analyzed from duplicate samples with initial TFA hydrolysis (2 N TFA at 121º C for 1 h) prior to reduction, acetylation and analysis by GC (**Englyst, Wiggins, & Cummings, 1984**) using inositol as an internal standard. Calibration was performed using a series of standard solutions of L-rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man) and D-xylose (Xyl). The chromatographic conditions were described by **Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent & Fernández-Bolaños** (2012).

Total phenolic content was determined using the Folin−Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents (**Singleton & Rossi, 1965**).

2.6 Relative molecular weight determination

The homogeneity and molecular weight distribution of the purified Pectoliv extracts were estimated by high performance size exclusion chromatography (HPSEC) using two different columns (300 X 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA) in sequence TSK gel GMPWXL (dextran MW<50000 kDa) and TSKgel G3000PWXL (dextran MW<60 kDa) as described previously (**Dos-Santos, Jiménez-Araujo, Rodríguez-Arcos, & Fernández-Trujillo, 2011**). The system was calibrated with standard dextrans of 500, 110, 70, 40, and 6 kDa using a regression curve.

2.7 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is based upon the inhibition of peroxyl radical-induced oxidation initiated by the thermal decomposition of 2, 2'-azobis(2-amidino-propane) dihydrochloride (AAPH). The reactive oxygen species (ROS) generated from this thermal decomposition quench the signal from the fluorescent probe fluorescein. The antioxidant capacity of the samples was assayed according to **Ou, Hampsch-Woodill, & Prior** (2001) with minor modifications. Samples were diluted with sodium phosphate buffer (10 mM, pH 7.4) and 25 µL of each were transferred to a microplate. The blank well received 25 µL phosphate buffer while standards received 25 µL Trolox solutions (10-140 µM). Then 150 µL of 1 µM fluorescein was added to all wells. After incubation (37º C, 15 min), 25 µL AAPH (250 mM) was added to each well to initiate the reaction and readings were taken every 5 min for 90 min (Ex. 485 nm, Em. 538 nm) in a microplate reader (Fluoroskan AscentTM, Thermo ScientificTM). Results were calculated using the difference of areas under the fluorescein decay curve between the blank and the sample and expressed as µM Trolox equivalents.

2.8 Cell culture and treatment

Caco-2 and THP-1 cells were cultured and treated as described (**Bermúdez-Oria et al, 2019b**). Cells were cultured in Dulbecco's Modified Eagle Medium (1000 mg/ml glucose, 110 mg/ml pyruvate, and 580 mg/ml glutamine) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100 U/ml penicillin, and 100 g/ml streptomycin and incubated at 37º C with 5% CO2. Fetal bovine serum was heat-inactivated at 56° C for 30 minutes. Caco-2 cells were subcultured once a week using trypsin-ethylenediaminetetraacetic acid, and medium was replenished once between passages. THP-1 cells were subcultured every 2–3 days by resuspension in fresh medium. Treatments were carried out under the same standard culture conditions. The lyophilized Pectoliv extracts were dissolved in Hank's Balanced Salt Solution (HBSS) at 100 mg/ml, heated at 100º C for 30 minutes, and diluted with culture medium as required. Cells were seeded in 96-well microplates (4 x 104 or 14 x 104 cells/well, 50 µl/well). Extracts were

added to the same volume (50 µl/well) to achieve a final concentration of 10% v/v HBSS. Caco-2 and THP-1 cells were incubated for up to 9 and 4 days, respectively. In addition to the cell proliferation assay (described in *2.9*), cells were periodically inspected under the phase contrast microscope.

2.9 Cell proliferation assays

Proliferation of adherent (Caco-2) and suspended (THP-1) cells was determined by measuring proliferation at different times using the neutral red and thiazolyl blue tetrazolium bromide (MTT) assays, respectively. For the neutral red assay, cells in 96-well plates were incubated in fresh culture medium containing the vital stain neutral red (50 µg/ml) for 30 minutes. Cells were then washed using HBSS, and the stain was extracted using acetic acid (75 µl, 1% (v/v) in ethanol 50% (v/v)). Absorbance was measured at 550 nm using a plate reader (**Borenfreund & Puerner, 1985; Girón-Calle, Alaiz, & Vioque, 2010**). For the MTT assay, cells in 96-well plates were incubated in culture medium containing MTT (0.5 mg/ml) for 60 minutes. The blue formazan crystals formed by the reduction of MTT were dissolved by the addition of 100 µL HCl (0.1 N) in isopropanol, and absorbance at 570 nm (with subtraction at 630 nm) was measured using a plate reader (**Kops, West, Leach, & Miller, 1997; Girón-Calle et al., 2010**).

3. Results

3.1. Preparation of the extracts from alperujo

Five extracts were obtained from olive oil by-product by different thermal and chemical treatments. Pectoliv-80 was obtained from a mild thermal treatment, using the conventional temperature for the recovery of pectin from fruits, 80º C for 60 min. Extracts Pectoliv-120 and Pectoliv-160 were obtained from thermal treatments at 120º C for 60 min and 160º C for 30 min respectively, with the objective of reducing the molecular weight to gain a product similar to a heat-modified water-soluble pectin preparation, which presents a known antiproliferative effect (**Lefsih et al., 2018**).

In order to obtain other extracts of low molecular weight by chemical treatment, Pectoliv-80 and Pectoliv-120 were hydrolyzed with alkali (NaOH). The resulting products were named Pectoliv-80A and Pectoliv-120A, respectively. The same chemical treatment is used for production of PectaSol-C, a commercial modified citrus pectin (MCP) extracted from citrus pectin (**Jackson et al., 2007**), although some authors indicate that MCP may be enzymatically modified (**Maxwell et al., 2012**). The latter extracts were modified in a variety of ways to provide an array of extracts with varying chemical composition and molecular weight.

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3.2 Effect of the olive extracts (Pectoliv) and MCP on cell proliferation of Caco-2 cells

The effect of each of the five different extracts – differing in their method of preparation, thermal or chemical treatment – on the proliferation of Caco-2 colon cancer cells was investigated to understand whether the different extraction methods affected the extracts' biological effect. Cells were treated with 0.35, 1.10. 3.50 and 10 mg/mL of Pectoliv extracts or commercial MCP for up to 7 days. At 0.35 and 1.10 mg/mL, neither the five Pectoliv extracts nor MCP inhibited cell proliferation (**Figure 2**). Incubation with 3.5 mg/mL Pectoliv-80 inhibited cell proliferation completely, while the inhibition caused by Pectoliv-80A was not as high. Pectoliv-80, -80A, -120 and -160 completely inhibited cell proliferation at 10 mg/mL from the first day of treatment. Pectoliv-120A and MCP reduced proliferation to a minor extent, reaching a maximum inhibition of 75% and 56% at day 7, respectively. Several studies indicate that pectins with a lower molecular weight, including Pectasol-C, exhibit greater bioactivity than pectins with a higher molecular weight (**Hayashi, Gillen, & Lott, 2000; Maxwell et al., 2012**). However, our results show that Pectoliv-80 has a higher inhibitory effect than Pectoliv-80A, which has suffered hydrolysis by treatment with alkali. This result suggests that molecular weight alone is not indicative of bioactivity.

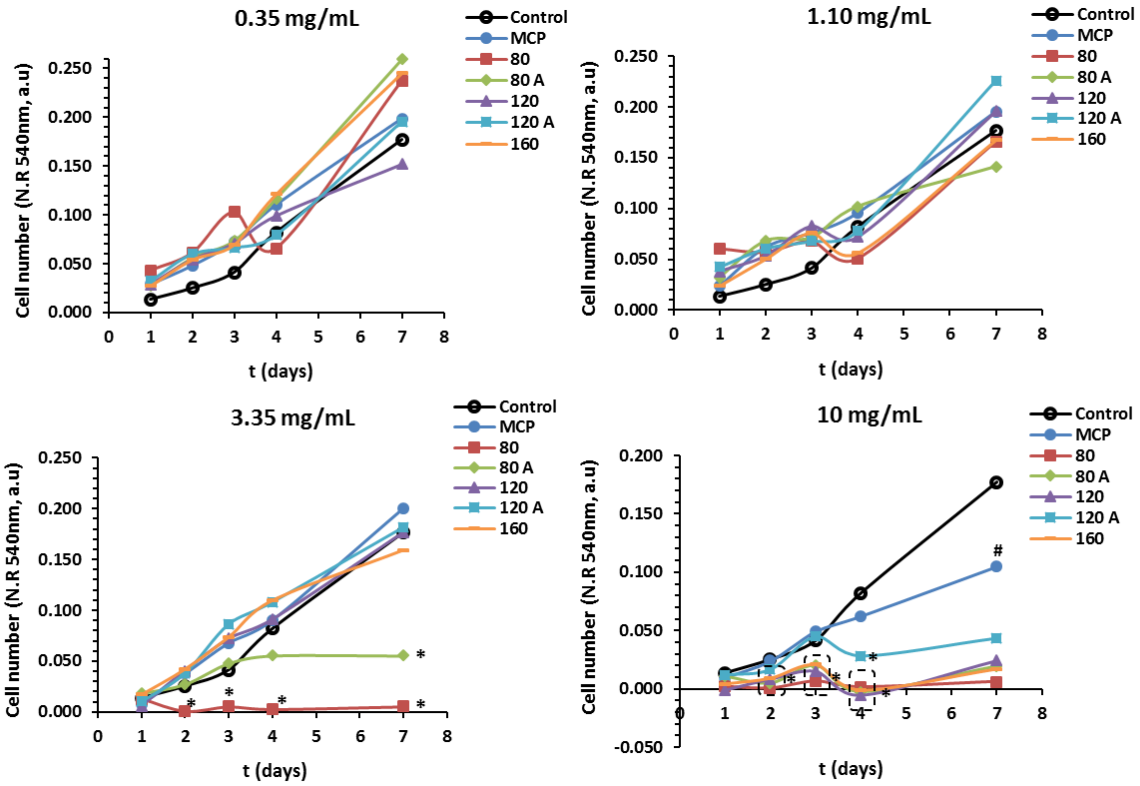

Figure 2. Anti-proliferative activity of the extracts obtained by thermal (Pectoliv-80, -120 and -160) or thermal and chemical (Pectoliv-80A and -120A) treatment at four different concentration, as compared to MCP. Neutral red uptake was determined after incubation of Caco-2 cells for 1 to 7 days. Data are the mean of three replicates ± SD. * indicate statistical significant difference between sample and vehicle control for each incubation period and # indicate statistical difference of the all samples for the day of incubation (one way ANOVA-LSD test, $p<0.05$).

3.3. *Antioxidant activities of Pectoliv and MCP*

The antioxidant activity of the five Pectoliv extracts and MCP were evaluated according to their oxygen radical absorbance capacity (ORAC). The five Pectoliv extracts showed higher antioxidant capacity (with values between 484 and 1342 µmol/ g extract) than the commercial MCP (314 µmol/ g extract) (**Figure 3**). These results were similar to those obtained in previous studies with other Pectoliv extracts (**Bermúdez-Oria et al., 2019**). Pectoliv-80 showed the highest antioxidant activity, followed by Pectoliv-80A, which can be explained because these extracts contained two-fold more phenolic

compounds than the other extracts, as seen in the chemical characterization (Table 1). The ORAC assay confirmed that these phenols partially maintained their antioxidant activity after their theoretical polymerization and binding to the polysaccharide fraction, which indicates certain availability of the catechol groups, responsible for antioxidant activity (**Spizzirri et al., 2009**). These results coincide with those obtained from the cell proliferation assay in Caco-2 cells, where a direct relationship between a major antiproliferative activity for Pectoliv-80 and Pectoliv-80A and their higher antioxidant activity was observed. These findings are also in agreement with those of other authors who published similar observations with different degrees of potency depending on the type of cancerous cell line and antioxidant extract studied (**Seeram et al., 2006; Tow, Premier, Jing, & Ajlouni, 2011; Baby, Antony, & Vijayan, 2017; Losada-Echeberria, Herranz-Lopez, Micol, & Barrajon-Catalan, 2017**). In addition, although MCP was recently shown to be an effective antioxidant (**Hawach, Boujaoude, & Abdel-Massih, 2016; Ramachandran, Wilk, Malnick, & Eliaz, 2017**), our data, using the ORAC assay, showed that MCP had a lower antioxidant activity than the Pectoliv extracts, which also coincided with the lowest antiproliferative activity. Effectively, the Pectoliv extracts have important antioxidant properties, including free-radical scavenging, acting as hydrogen donors to the peroxyl radical generated in the ORAC assay. Peroxyl radicals are reactive species generated during lipid oxidation in food and living tissues (**Lobo, Patil, Phatak, & Chandra, 2010; Phaniendra, Jestadi, & Periyasamy, 2015**). Thus, these ORAC values can be considered to be of biological relevance as a reference of antioxidant effectiveness. Therefore, there appears to be a direct relationship between the antioxidant activity of each extract and its antiproliferative activity.

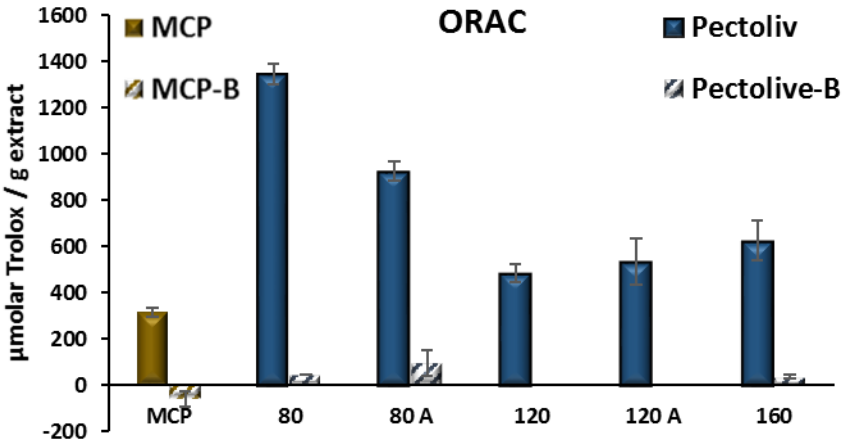

Figure 3. Antioxidant capacity of Pectoliv extracts (unbleached and bleached extracts) compared to those for commercial modified citrus pectin (MCP). Oxygen radical capacity (ORAC) is expressed as µmol Trolox/g extract. Average, and standard deviations are shown (n = 3).

3.4. Chemical composition and molecular weight of the Pectoliv extracts

The uronic acid content of the extracts increased with temperature up to 120º C/ 60 min, with a slight decrease at 160º C/ 30 min (**Table 1**). However, alkali treatment did not affect the uronic acid content or reduce the molecular weight, as might be expected by chemical β elimination (**Renard & Thibault, 1996**). Indeed, certain depolymeration with the reduction of molecular weight was only observed when the treatment temperature was increased (**Figure 4**). The ratio of uronic acids to neutral sugars was between 1 and 1.5 for different Pectoliv extracts, which is consistent with previous results reported by **Rubio-Senent et al**., (2015a), compared to a ratio of 9 for MCP. The US Pharmacopeia defines pectin as a polysaccharide with not less than 74% of galacturonic acid (**Rolin, 2002**), yet all characterized Pectoliv extracts had a content of 49%–61% uronic acids and so cannot be considered as pectins. However, Pectoliv extracts had a high proportion of uronic acids so they could be considered as "pectin-like polysaccharides" with a high proportion of associated neutral sugars and phenols.

Table 1. Chemical composition (g/100 g) and glycosyl residue composition (% molar ratio) of Pectoliv extracts obtained by thermal (Pectoliv-80, -120 and -160) or thermal and chemical (Pectoliv-80A and -120A) treatment as compared to modified citrus pectin (MCP). The data shown are mean ± standard deviation. Rha: rhamnose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; UrA: uronic acids. N.D. not detected.

		Pectoliv					
	MCP	80	80 A	120	120 A	160
Uronic acid	51.52 ± 7.78	26.98 ± 2.6	29.93 ± 0.92	42.29 ± 1.87	48.51 ± 1.27	38.67 ± 0.59
Neutral sugar	5.24 ± 0.07	23.33 ± 3.00	30.22 ± 0.92	28.78 ± 0.90	29.92 ± 0.60	36.28 ± 1.93
Phenol	0.15 ± 0.01	8.08 ± 0.1	10.93 ± 0.32	4.54 ± 0.13	4.71 ± 0.06	4.93 ± 0.08
Total	56.91	58.39	71.08	75.61	83.14	79.88
UrA	90.77 ± 0.35	51.46 ± 0.00	49.22 ± 0.00	58.97 ± 0.00	61.51 ± 0.00	50.67 ± 0.00
Rha	1.69 ± 0.11	4.47 ± 0.11	5.82 ± 0.36	5.49 ± 1.97	6.03 ± 0.37	6.28 ± 0.16
Fuc	N.D	0.22 ± 0.01	0.31 ± 0.02	0.21 ± 0.04	0.22 ± 0.00	0.32 ± 0.01
Ara	1.08 ± 0.14	22.62 ± 2.30	23.11 ± 0.28	12.50 ± 0.98	11.00 ± 0.31	14.44 ± 0.52
Xyl	0.49 ± 0.04	1.93 ± 0.13	2.12 ± 0.05	4.21 ± 0.24	3.75 ± 0.09	5.84 ± 0.21
Man	0.56 ± 0.07	1.70 ± 0.08	1.82 ± 0.03	2.49 ± 0.29	2.44 ± 0.04	3.64 ± 0.18
Gal	4.67 ± 0.08	11.97 ± 0.82	13.36 ± 0.07	12.15 ± 0.51	11.29 ± 0.35	14.60 ± 0.56
Glc	0.63 ± 0.01	3.37 ± 0.26	3.70 ± 0.05	3.45 ± 0.18	3.41 ± 0.06	3.28 ± 0.01

The composition of neutral sugars showed that the extracts were rich in arabinose and galactose, with rhamnose, xylose and glucose present in minor proportions, which is indicative of arabinogalactan being an integral, branched region of pectin. The phenol content was higher in Pectoliv-80 and -80A, with 8% and 11% respectively, more than double the amount of the other extracts, while the phenol content for MCP was very low, at only 0.15%. The presence of these phenolic compounds could be due to the possible interaction between the polysaccharides of the olive cell wall and the hydrophilic compounds, such as phenols, which occurs during the rupture of fruit tissues by olive crushing and further malaxation of the olive paste before oil extraction. In addition, oxidation, condensation and/or polymerization reactions (via enzymatic or non-enzymatic action) provide a significant quantity of non-carbohydrate polymeric material associated with the cell wall polysaccharides (**Obied, Allen, Bedgood, Prenzler, & Robards, 2005**). These findings in Pectoliv-80 and -80A were probably due to their high polyphenol concentration (since polyphenols bind to pectin polysaccharides), and correlate with their high antioxidant and antiproliferative activity.

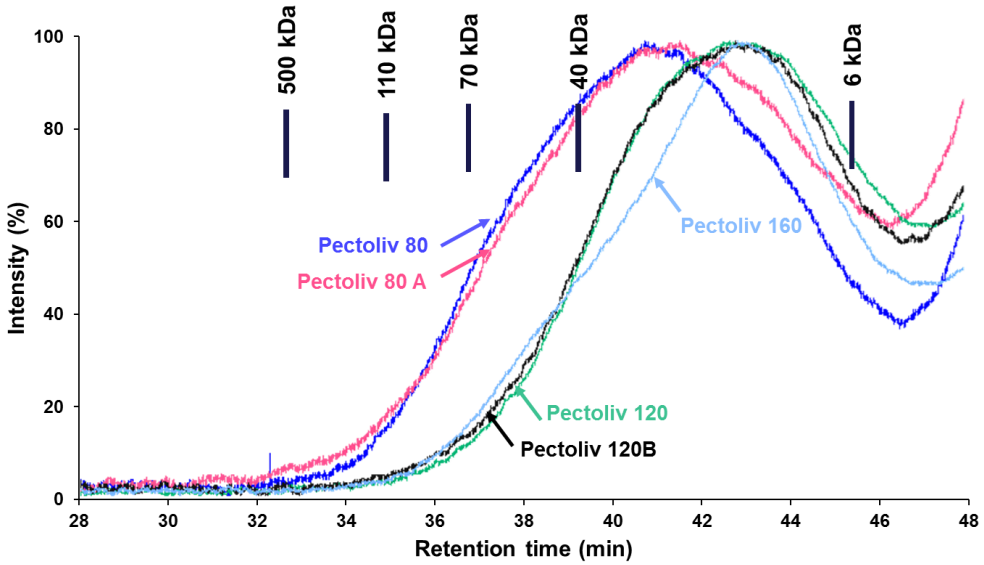

Figure 4. Refractive index elution profiles of the Pectoliv extracts obtained by high performance size exclusion chromatography using two TSK gel columns (GMPWXL and G3000PWXL) placed in a series. Numbers above the peaks represent molar masses of dextran standards.

3.5. Bleaching of Pectoliv extracts: chemical composition and antioxidant activity

Due to the presence of chemical linkages between polyphenols and carbohydrates, pectin-bound polyphenols are difficult to eliminate. In order to confirm the components of the extracts responsible for their antiproliferative activity, polyphenols in Pectoliv-80, -80A and -160, and MCP, were eliminated by bleaching using sodium chlorite in acid medium. The resulting low-molecular weight oxidized products were eliminated by dialysis. The bleached extracts were characterized and their antioxidant activities measured. Although the phenolic compounds were degraded or solubilized, in accordance with Gellerstedt (2009), the bleached extracts did not show a substantial modification in uronic acid or sugar composition (**Table 2**). The ratio of uronic acids to neutral sugars (0.7–2) was very similar to unbleached Pectoliv for MCP-B. The uronic acid content in the bleached samples varied between 24% and 56%, with a decrease in the MCP-B (37%) with respect to MCP (52%) or an increase from 39% to 56% for Pectoliv-160-B.

Tabla 2. Chemical composition (g/100 g) and glycosyl residue composition (% molar ratio) of bleached Pectoliv extracts (Pectoliv-B) and commercial modified citrus pectin (MCP-B). The data shown are mean ± standard deviation. Rha: rhamnose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; UrA: uronic acids. N.D. not detected.

		Pectoliv-B		
	MCP-B	80	80 A	160
Uronic acid	37.03 ± 4.94	29.41 ± 2.83	23.79 ± 2.45	56.45 ± 1.51
Neutral sugar	4.52 ± 0.51	29.54 ± 3.20	33.71 ± 5.10	24.82 ± 1.78
Phenol		N.D		
Total	41.55	58.95	57.50	81.27
UrA	87.33 ± 2.64	49.45 ± 6.02	41.53 ± 4.96	69.49 ± 1.40
Rha	3.56 ± 1.68	5.29 ± 0.97	5.18 ± 1.52	5.47 ± 0.29
Fuc	0.29 ± 0.05	0.36 ± 0.01	0.36 ± 0.08	0.32 ± 0.01
Ara	1.61 ± 0.09	23.42 ± 2.71	24.83 ± 1.16	7.88 ± 0.22
Xyl	0.94 ± 0.09	1.71 ± 0.13	6.96 ± 8.75	2.97 ± 0.13
Man	0.38 ± 0.13	2.46 ± 0.03	2.09 ± 0.32	2.21 ± 0.82
Gal	3.59 ± 0.48	14.21 ± 1.95	15.84 ± 0.90	10.04 ± 0.23
Glc	2.31 ± 0.31	3.09 ± 0.28	3.22 ± 0.32	1.63 ± 0.02

Bleaching caused a drastic decrease in the antioxidant capacity of Pectoliv extracts (**Figure 3**), reducing the ORAC activity from 484–1342 µmol/ g extract to 34–94 µmol/ g extract, and with ORAC disappearing completely in MCP-B. These results suggest a clear correlation between antioxidant activity and the presence or absence of the phenolic compounds in the Pectoliv extracts.

3.6. Effect of bleached Pectoliv extracts on the proliferation of Caco-2 and THP-1 cell lines

In order to assess the correlation between the presence of phenolic compounds in the extracts and their antiproliferative effect on cells, the Caco-2 colon cancer and THP-1 leukemia cancer cell lines were treated with bleached extracts. Following the exposure of Caco-2 cells to increasing concentrations (2.5, 5 and 10 mg/mL) of bleached and unbleached Pectoliv extracts (-80-B, -80AB, -160-B and -80, -80A, -160, respectively), we observed that at 2.5 mg/mL, and more clearly at 5 mg/mL (**Figure 5A**), all the unbleached Pectoliv extracts inhibited cell proliferation compared to the bleached samples and the control, with a clear time dependent effect. It is remarkable that the inhibitory activities of bleached Pectoliv were similar to bleached and unbleached MCP for the same concentration and the same treatment time. This effect was even more evident at higher concentrations. For example, at 10 mg/mL, Pectoliv-80, -80A, and -160 completely inhibited proliferation after 1 or 2 days of treatment (with a continued inhibition of proliferation from there on), while the corresponding bleached Pectoliv extracts allowed proliferation to a similar extent as MCP and MCP-B.

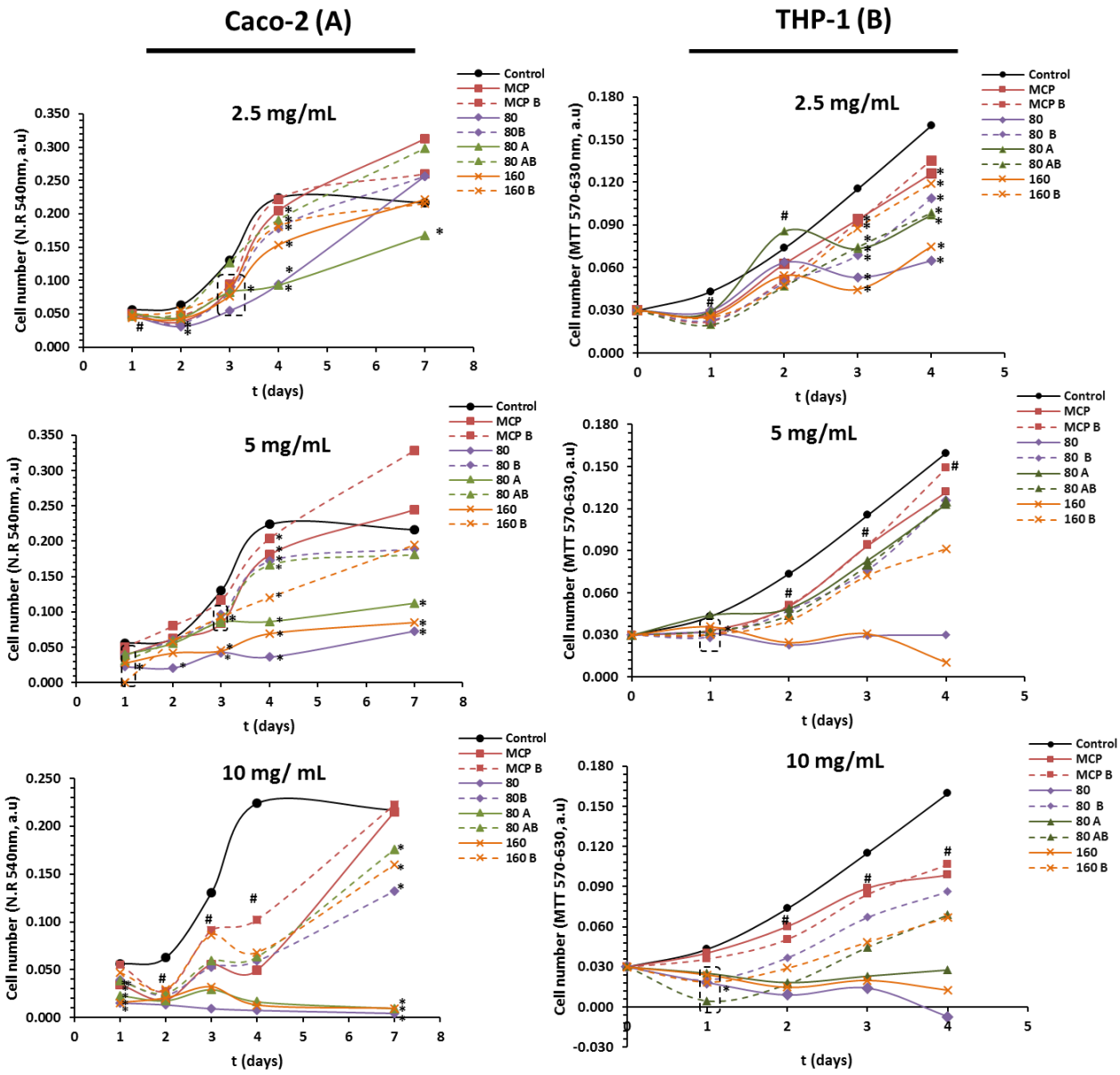

Figure 5. Effect of unbleached and bleached Pectolive extracts on proliferation of (A) Caco-2 and (B) THP-1 cells. Cells were cultured in 96-well plates (5 x 10^3 cells/well) in culture medium containing 2.5, 5 and 10 mg/mL of extract (Pectoliv-80, -80A, -160, and MCP unbleached and bleached) and a control (without extract). For the Caco-2 cells, Uptake of neutral red by Caco-2 cells was determined after incubation for 1, 2, 3, 4 or 7 days, and reduction of MTT by THP-1 cells was determined after incubation for 1, 2, 3 or 4 days. Data represent the mean of three incubations ± SD. * indicate statistical significant difference between sample and vehicle control for each incubation period, #

indicate statistical difference of the all samples for the day of incubation (one way ANOVA-LSD test, $p<0.05$).

Next, THP-1 cells were treated with the same range of concentration of bleached and unbleached extracts for 4 days. Treatment with 2.5 mg/ mL unbleached Pectoliv-80 and -160 resulted in reduced cell proliferation with respect to the control, and almost completely inhibited proliferation at 5 mg/ mL (**Figure 5B**). Similar to the results with Caco-2 cells, bleaching of these Pectolivs greatly reduced the antiproliferative activity. At 10 mg/ mL, proliferation was almost completely inhibited by the three unbleached Pectoliv extracts. At this concentration, bleaching of these three Pectolivs allowed for a proliferation rate similar, although still lower, than the proliferation of THP-1 cells exposed to either bleached or unbleached MCP.

Therefore, elimination of phenols by bleaching greatly reduced the antiproliferative activity of Pectolivs on both Caco-2 and THP-1 cells, resulting in an effect similar or superior to the antiproliferative effect of MCP. Consequently, the phenols linked to polysaccharides seem to be responsible for the majority of the antiproliferative effect observed for the Pectoliv extracts isolated from the by-product of olive oil extraction. Our findings are consistent with reports showing an antiproliferative effect of polyphenols or polyphenol-rich extracts on Caco-2, THP-1 and other cancer cell lines (**Tsan, White, Maheshwari, Bremner, & Sacco, 2000; Girón-Calle et al., 2004; Megías et al. 2009; Gul, Ahmad, Kondapi, Qureshi, & Ghazi, 2013; Sánchez-Vioque et al., 2016**). In addition, a polyphenol mixture from olive oil processing waste was described to decrease the production of superoxide anion in THP-1 cells (**Léger, Kadiri-Hassani, & Descomps, 2000**). Since high levels of ROS are present in cancer cells and may help tumor survival by inactivation of the caspases involved in apoptosis induction (**Halliwel, 2007**), the Pectoliv extracts, via their free-radical scavenging capacity, could help to reduce the concentrations of ROS and induce apoptosis.

4. Conclusions

This study revealed that the Pectoliv extracts had a high antioxidant and antiproliferative activity on Caco-2 and THP-1 cell lines, as compared to MCP. Interestingly, the extracts obtained at the lowest temperature, Pectoliv-80 and Pectoliv-80A, had the highest antiproliferative and antioxidant activity, consistent with them having the highest content of phenolic compounds. Bleaching caused an important decrease in the antiproliferative activity of all Pectoliv extracts, reaching levels similar to those found for MCP. Moreover, bleaching produced a drastic decrease in the antioxidant activity of the Pectoliv extracts. Therefore, we showed that the high antioxidant and antiproliferative activity observed in the extracts from alperujo was mostly due to their content of phenolic compounds. However, other components in these extracts, possibly pectic polysaccharides, may also be involved in the antiproliferative activity, as indicated by the fact that the antiproliferative activity of bleached Pectolivs was still similar or even superior to that of commercial MCP. These Pectolivs are products with a high potential for application in the pharmaceutical industry.

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Declarations of interest

None.

References

Baby, B., Antony, P., & Vijayan, R. (2017). Antioxidant and anticancer properties of berries. *Critical Reviews in Food Science and Nutrition*, DOI: 10.1080/10408398.2017.1329198.

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Fernández-Bolaños, J, & Sánchez-Carbayo, M. (2019a). Olive extract rich in polysaccharides with antioxidant and antiproliferative activity on bladder cancer cells. *Manuscript submitted for publication*.

Bermúdez-Oria, A., Rodríguez-Gutiérrez, G., Alaiz, M., Vioque, J., Girón-Calle, J., & Fernández-Bolaños, J. (2019b). Pectin-rich extracts from olives inhibit proliferation of Caco-2 and THP-1 cells. *Manuscript submitted for publication*.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*(2), 484–489.

Cardoso, S. M., Coimbra, M. A., & Lopes da Silva, J.A. (2003). Calcium-mediated gelation of an olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Polymers, 52*, 125- 133.

Dos-Santos, N., Jiménez-Araujo, A., Rodríguez-Arcos, R., & Fernández-Trujillo, J. P. (2011). Cell Wall Polysaccharides of Near-Isogenic Lines of Melon (*Cucumis melo* L.) and Their Inbred Parentals Which Show Differential Flesh Firmness or Physiological Behavior. *Journal of Agricultural and Food Chemistry*, *59*(14), 7773–7784.

Englyst, H., Wiggins, H. S., & Cummings, J. H. (1982). Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. *The Analyst*, *107*(1272), 307.

Fernández-Bolaños, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Sánchez, P. Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de oliva. ES2374675. 2010.

Gellerstedt, G. (2009). Chemistry of bleaching of chemical pulp. In M. Ek, G. Gellerstedt, & G. Henriksson (Eds), *Pulp and Paper Chemistry and Technology* (pp. 201-237). Berlin: Walter de Gruyter GmbH & Co. KG.

Girón-Calle, J., Alaiz, M., & Vioque, J. (2010). Effect of chickpea protein hydrolysates on cell proliferation and in vitro bioavailability. *Food Research International, 43*, 1365–1370.

Gul, M. Z., Ahmad, F., Kondapi, A. K., Qureshi, I. A., & Ghazi, I. A. (2013). Antioxidant and antiproliferative activities of Abrus precatorius leaf extracts - an in vitro study. *BMC Complementary and Alternative Medicine*, *13*, 53.

Halliwell, B. (2007). Oxidative stress and cancer: have we moved forward? *Biochemistry Journal, 401*, 1–11.

Hawach, V., Boujaoude, M. A., & Abdel-Massih, R. M. (2016). The cytotoxic and anti-proliferative activity of high molecular weight pectin and modified citrus pectin. *Functional Foods in Health and Disease, 6(9),* 587-601.

Hayashi, A., Gillen, A. C., & Lott, J. R. (2000). Effects of daily oral administration of quercetin chalcone and modified citrus pectin on implanted colon-25 tumor growth in Balb-c mice. *Alternative Medicine Review, 5*(6) 546–552

Jackson, C. L., Dreaden, T. M., Theobald, L. K., Tran, N. M., Beal, T. L., Eid, M., … Mohnen, D. (2007). Pectin induces apoptosis in human prostate cancer cells: correlation of apoptotic function with pectin structure. *Glycobiology*, *17*(8), 805–819.

Kops, S. K., West, A. B., Leach, J., & Miller, R. H. (1997). Partially purified soy hydrolysates retard proliferation and inhibit bacterial translocation in cultured C2BBe cells. *Journal of Nutrition, 127*(9), 1744–1751.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids*, *28*(1), 92–104.

Lefsih, K., Giacomazza, D., Passantino, R., Costa, M. A., Bulone, D., Mangione, M. R., … Madani, K. (2018) Biochemical and biophysical characterization of water-soluble pectin from Opuntia ficus-indica and its potential cytotoxic activity. *Phytochemistry, 154*, 47-55.

Léger, C. L., Kadiri-Hassani, N., & Descomps, B. (2000). Decreased superoxide anion production in cultured human promonocyte cells (THP-1) due to polyphenol mixtures from olive oil processing wastewaters. *Journal of Agricultural and Food Chemistry*, *48,* 5061–5067.

Liu, H. Y., Huang, Z. L., Yang, G. H., Lu, W. Q., & Yu, N. R. (2008) Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model. *World Journal of Gastroenterology, 14* (48), 7386–7391.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4(8),* 118–126.

Losada-Echeberria, M., Herranz-Lopez, M., Micol, V., & Barrajon-Catalan, E. (2017). Polyphenols as Promising Drugs against Main Breast Cancer Signatures. *Antioxidants, 6*, 88.

Maxwell, E. G., Belshaw, N. J., Waldron, K. W., & Morris, V. J. (2012). Pectin - an emerging new bioactive food polysaccharide. *Trends in Food Science & Technology, 24*(2), 64–73.

Maxwell, E. G., Colquhoun, I. J., Chau, H. K., Hotchkiss, A. T., Waldron, K. W., Morris, V. J., & Belshaw, N. J. (2016). Modified sugar beet pectin induces apoptosis of colon cancer cells via an interaction with the neutral sugar side-chains*. Carbohydrate Polymers, 136*, 923–929.

Megías, C., Pastor-Cavada, E., Torres-Fuentes, C., Girón-Calle, J., Alaiz, M., Juan, R., Pastor, J., & Vioque, J. (2009). Chelating, antioxidant and antiproliferative activity of Vicia sativa polyphenol extracts. *European Food Research and Technology, 230*, 353–359.

Mudgil, D., & Barak, S. (2013). Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: A review. *International Journal of Biological Macromolecules, 61*, 1–6.

Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., & Raz, A. (2002). Inhibition of human cancer cell growth and metastasis in nude mice by

oral intake of modified citrus pectin. *Journal of the National Cancer Institute*, *94*(24), 1854–1862.

Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., & Robards, K. (2005). Investigation of australian olive mill waste for recovery of biophenols. *Journal of Agricultural and Food Chemistry*, *53,* 9911–9920.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry, 4*9, 4619–4626.

Phaniendra A., Jestadi D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry, 30(1),* 11–26.

Ramachandran, C., Wilk, B., Melnick, S. J., & Eliaz, I. (2017). Synergistic Antioxidant and Anti-Inflammatory Effects between Modified Citrus Pectin and Honokiol. *Evidence-Based Complementary and Alternative Medicine, 2017*, 1–9.

Renard, C.M.G.C., & Thibault, J.-F. (1996). Pectins in mild alkaline conditions: β-elimination and kinetics of demethylation. *Progress in Biotechnology, 14*, 603–608.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science and Technology, 17*, 3–15.

Rolin, C. (2002). Commercial pectin preparation. G. B. Seymour, & J. P. Knox (Eds), In *Pectin and their manipulation* (pp. 222-230). Oxford: Blackwell Publishing,

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A. & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. *Journal of Agricultural and Food Chemistry, 60,* 1175–1186.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2013). Phenolic extract obtained from steam-treated olive oil waste:

Characterization and antioxidant activity. *LWT- Food Science and Technology, 54*, 114–124.

Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Munoz, A., & Fernandez-Bolaños, J. (2015a). Pectin extracted from thermally treated olive oil by-products: Characterization, physico-chemical properties, in vitro bile acid and glucose binding. *Food Hydrocolloids, 43*, 311–321. doi: 10.1016/j.foodhyd.2014.06.001

Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Munoz, A., Garcia, A., & Fernandez-Bolaños, J. (2015b). Novel pectin present in new olive mill wastewater with similar emulsifying and better biological properties than citrus pectin. *Food Hydrocolloids, 50*, 237–246. doi: 10.1016/j.foodhyd.2015.03.030.

Sánchez-Vioque, R., Santana-Méridas, O., Polissiou, M., Vioque, J., Astraka, K., Alaíz-Barragán, M., … Girón-Calle, J. (2016). Polyphenol composition and in vitro antiproliferative effect of corm, tepal and leaf from Crocus sativus L. on human colon adenocarcinoma cells (Caco-2). *Journal of Functional Foods, 24*, 18–25.

Seeram, N. P., Adams, L., Zhang, Y., Rupo, L., Sand, D., Scheuller, H., & Heber, D. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry extracts inhibit growth stimulate apoptosis of human cancer in vitro. *Journal of Agricultural and Food Chemistry, 54*, 9329–9339.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, *16*(3), 144–158.

Spizzirri, U. G., Iemma, F., Puoci, F., Cirillo, G., Curcio, M., Paris, O. I., & Picci, N. (2009). Synthesis of antioxidant polymers by grafting of gallic acid and catechin on gelatin. *Biomacromolecules, 10(7),* 1923–1930.

Tow, W. W., Premier, R., Jing, H., & Ajlouni, S. (2011). Antioxidant and antiproliferation effects of extractable and nonextractable polyphenols isolated from apple waste using different extraction methods. *Journal of Food Science, 76(7),* 163–172.

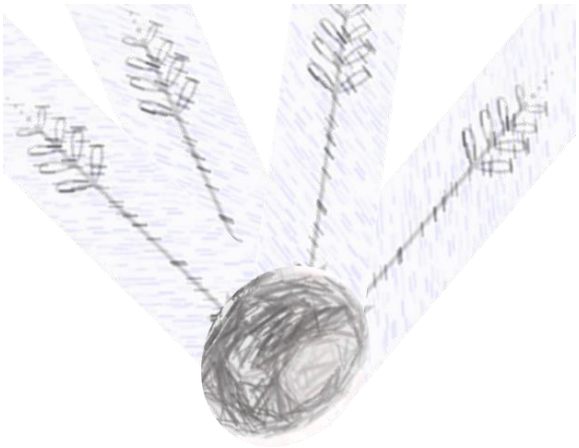
Tsan, M. F., White, J. E., Maheshwari, J. G., Bremner, T. A., & Sacco, J. (2000). Resveratrol induces Fas signalling-independent apoptosis in THP-1 human monocytic leukaemia cells. *British Journal of Haematology, 109(2),* 405–412.

Wollowski, I., Rechkemmer, G., & Pool-Zobel, B. L (2001). Protective role of probiotics and prebiotics in colon cancer. *The American Journal of Clinical Nutrition, 73(2*), 451S–455S.

Zhang, W., Xu, P., & Zhang, H. (2015). Pectin in cancer therapy: A review. *Trends in Food Science and Technology, 44,* 258–271.

Zhang, W. B., Gao, L., Shi, X. F., & Zhang, Q. L (2007). Determination of the molecular mass of modified citrus pectin using high performance size exclusion chromatography. *Chinese Journal of Chromatography, 25*(5), 711–714.

6. Conclusiones



6. Conclusiones

1.- Se ha conseguido encapsular mediante formulación en base de pectina, los dos antioxidantes de la aceituna, HT y DHFG, en cantidades suficientes para alcanzar el colon, comprobándose la formación de un complejo pectina-fenol, el cual mantiene parte de la actividad antioxidante de los fenoles, así como su efecto antiinflamatorio *in vivo*.

2.- Se han conseguido formular películas y recubrimientos comestibles con los dos antioxidantes HT y DHFG, presentando buenas propiedades fisicoquímicas, y protegiendo del ataque de hongos y evitando la peroxidación de la grasa de los alimentos.

3.- Se ha conseguido funcionalizar fibra alimentaria (dietaria) de fresa y manzana con los antioxidantes HT y DHFG, mediante la formación de un complejo fibra-fenol resistente, que mantiene en parte la actividad antioxidante de los fenoles y que combina los efectos beneficiosos tanto de la fibra como de los antioxidantes.

4.- Se han obtenido extractos ricos en pectinas modificadas con polifenoles asociados a partir de los alperujos y que presenta una alta actividad antiproliferativa in vitro sobre líneas celulares de cáncer de vejiga (las epitelio transicionales RT112, J82, T24, y la escamosa ScaBER), de cáncer de colon (Caco-2) y de leucemia monocítica (THP-1), junto con una alta actividad antioxidante. Destacando sobremanera el nulo efecto antiproliferativo sobre células sanas, siguiendo el modelo de células Caco-2 confluentes diferenciadas a enterocitos. Los compuestos fenólicos presentes son los principales responsables de esta alta actividad biológica.

CURRICULUM VITAE

DATOS PERSONALES

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HISTORIAL ACADÉMICO

2009 – 2013 Grado en Química. Universidad de Sevilla. Calificación promedio (1-4) 2,53 (1-10) 8,36

2013 – 2014 Máster en estudios avanzados en Química. Universidad de Sevilla. Calificación promedio (1-4) 2,87 (1-10) 9,33

2014 –2015 Máster Universitario en Profesorado en Enseñanza Secundaria Obligatoria y Bachillerato, Formación Profesional y Enseñanzas de Idiomas (MAES). Especialidad Física y Química. Universidad de Sevilla (1-4)2,5 (1-10)8,42

2015- Actualidad. Realización de tesis doctoral Instituto de la Grasa (CSIC), departamento de Fitoquímica de alimentos. Sevilla. Beca FPI. **BES-2014-068508**

FORMACIÓN

Idiomas: Español. Lengua materna.

Inglés. Buena comprensión de textos escritos y nivel medio en conversación.
ISE II, certificado Trinity College London

Francés. Nivel medio de comprensión de textos escritos y nivel básico en conversación.

Conocimientos Informáticos: Alto conocimiento en los paquetes ofimáticos Microsoft Word y OpenOffice.

• **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Lama-Muñoz, A., & Fernández-Bolaños, J. (2017). Complexation of hydroxytyrosol and 3,4-dihydroxyphenylglycol with pectin and their potential use for colon targeting. *Carbohydrate Polymers 163*, 292–300.

• **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Vioque, B., Rubio-Senent, F., & Fernández-Bolaños, J. (2017). Physical and functional properties of pectin-fish gelatin films containing the olive phenols hydroxytyrosol and 3,4-dihydroxyphenylglycol. *Carbohydrate Polymers 178*, 368-377.

• **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Rodríguez-Juan, E., González-Benjumea, A., & Fernández-Bolaños, J. (2018). Molecular interactions between 3,4-dihydroxyphenylglycol and pectin and antioxidant capacity of this complex in vitro. *Carbohydrate Polymers. 197*, 260–268.

• **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Fernández-Prior, A., & Fernández-Bolaños, J. (2018). Effect of edible pectin-fish gelatin films containing the olive antioxidants hydroxytyrosol and 3,4-dihydroxyphenylglycol on beef meat during refrigerated storage. Meat Science. 148, 213-218. doi: 10.1016/j.meatsci.2018.07.003

•Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Rubio-Senent, F., **Bermúdez-Oria, A**., Fernandez-Prior. A & Fernández-Bolaños, J. (2018) Synergistic effect of 3,4-dihydroxyphenylglycol with hydroxytyrosol and α-tocopherol on the Rancimat oxidative stability of vegetable oils. *Innovative Food Science and Emerging Technologies, 51, 100-106 doi.org/10.1016/j.ifset.2018.08.001*

• Hernández-Hernández, C., Morales Sillero, A., Fernández-Bolaños, J., **Bermúdez-Oria, A.**, Azpeitia-Morales, A., Rodríguez-Gutiérrez, G. (2018). Cocoa bean husk: industrial source of antioxidant phenolic extract. *Journal of The Science of Food and Agriculture. 99*: 325–333 doi: 10.1002/jsfa.9191

- Voltes, A., **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Reyes, M. L., Olano, C., Fernández-Bolaños, J & de la Portilla, F. (2018). Anti-Inflammatory Local Effect of Hydroxytyrosol Combined with Pectin-Alginate and Olive Oil on Trinitrobenzene Sulfonic Acid-Induced Colitis in Wistar Rats". *Journal of Investigative Surgery. 15*, 1-7

- **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Fernández-Prior, A., Vioque, B., & Fernández-Bolaños, J (2018). Strawberry dietary fiber functionalized with phenolic antioxidants from olives. Interactions between polysaccharides and phenolic compounds. *Food Chemistry, 280*, 310-320. DOI: 10.1016/j.foodchem.2018.12.057

- Arandoa, A., Delgadoa, J.V., Fernández-Prior, A., León, J.M., **Bermúdez-Oria, A**., Nogales, S., Pérez-Marín, C.C. (2019) Effect of different olive oil-derived antioxidants (hydroxytyrosol and 3,4-dihydroxyphenylglycol) on the quality of frozen-thawed ram sperm. *Cryobiology, 86*, 33-39. *Doi: /10.1016/j.cryobiol.2019.01.002*

PARTICIPACIÓN EN PROYECTOS

AGL2013- 48291-R Recuperación de secoiridoides y fibra de efluentes de la industria oleícola. Formulación pectina-antioxidante. Evaluación de pectinas modificadas. IP Juan Fernández- Bolaños Guzmán. Ministerio de Economía, Industria y Competitividad.

AGL2016-79088-R Uso de disolventes eutécticos naturales para la extracción de fenoles y pectinas modificadas a partir de alperujo y aceite de oliva. Estudio de las propiedades biológicas de las pectinas modificadas. IP Juan Fernández-Bolaños Guzmán. Ministerio de Economía, Industria y Competitividad.

Proyecto d4-17. Antioxidante natural de origen vegetal para productos cárnicos altamente saludables. Ministerio de Economía, Industria y Competitividad.

CURSOS

- Curso Ciencia y Tecnología de Productos Detergentes (60 horas) (Centro de Formación Permanente de la Universidad de Sevilla).
- 16º Edición: Taller de operaciones básicas para el análisis de muestras por resonancia magnética nuclear de líquidos. 11 y 12 de noviembre 2015. Universidad de Sevilla. 6 horas y 15 minutos.
- Curso presentaciones “Disfrutar creando presentaciones efectivas” 16 horas
- Seguridad Biológica. UBC-IG. Servicio de prevención de riesgos laborales, CSIC. 31 de enero de 2018. 2 horas
- INGLÉS PRESENTACIONES por la Agencia Estatal Consejo Superior de Investigaciones Científicas e impartido por Euroconsultoría Formación Empresa, S.L. (Euroformac). Online. 01/10/2018 al 02/11/2018
- POWER POINT 2010 AVANZADO" organizado por la Agencia Estatal Consejo Superior de Investigaciones Científicas e impartido por Euroconsultoría Formación Empresa, S.L. (Euroformac). Online. 15/10/2018 al 19/11/2018.

SEMINARIOS - CONFERENCIAS

- Conferencia “Aprovechamiento integral del residuo industrial del aceite de palma: producción, desarrollo y aplicaciones de la celulosa nanofibrilar” 2h 10/02/15. Colegio de Químicos. Sevilla.
- Conferencia “El colesterol ¿amigo o enemigo?: Un repaso a su historia” 1.30h., 10/06/15. Colegio de Químicos. Sevilla.
- Conferencia “Enfermedades alimentarias: Histaminosis alimentaria no alérgica (HANA)”.,1.30h 08/10/15. Colegio de Químicos. Sevilla.

- Conferencia “Hitos de la química del suelo” 1.30h 15/12/15. Colegio de Químicos. Sevilla.
- Conferencia “Iminoazúcares como chaperonas farmacológicas para el tratamiento de enfermedades de almacenamiento lisosomal” 1 h 26/01/16. Colegio de Químicos. Sevilla.
- II Conferencia Internacional de la Red Chia-Link. 6-7 Octubre, 2016. CSIC-Instituto de la Grasa. Sevilla.
- Conferencia “Química, color y arte” 1,30 h 13/06/17. Colegio de Químicos. Sevilla.
- Jornada de difusión “**Investigación en valorización de biomasa derivada del olivar**”, organizada conjuntamente por el Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), la Universidad de Jaén y la Plataforma Tecnológica Española de la Biomasa (BIOPLAT). 18 de mayo de 2018.
- Conferencia “La física de la vida” 2 h 24/06/18. Colegio de Químicos. Sevilla

APORTACIÓN A CONGRESOS

- XVIII Simposio Científico-Técnico EXPOLIVA 2017. **Comunicación:** Obtención de compuestos biactivos mediante el uso combinado de tratamientos térmicos con nuevos disolventes eutécticos. 10-12 Mayo, 2017. Jaén. ISBN 978-84-946839-1-6

- XVIII Simposio Científico-Técnico EXPOLIVA 2017. **Comunicación**: Phenolic extract obtained from olive oil solid wastes thermally treated: Influence of pH. 10-12 Mayo, 2017. Jaén. ISBN 978-84-946839-1-6

- **1st International Congress on Metals in Anaerobic Biotechnologies (IMAB17)**. 4-6 Octubre 2017. Sevilla.

Short oral presentation and poster: Thermal treatment for olive oil wastes utilization: bioactive compounds and substrate for soil contaminated

remediation. G. Rodríguez Gutiérrez**,** J. Fernández-Bolaños Guzmán, A. Lama-Muñoz. F. Rubio-Senent, A. Fernández-Prior, E. María Rodríguez Juan, A. García Borrego, **A. Bermúdez Oria**, B. Vioque-Cubero.

- **1st Congreso de Estudiantes de Doctorado en Química**. Facultad de Química 12-13 diciembre 2017.

Comunicación oral: Pectinas modificadas de aceitunas con actividad antiproliferativa en células cancerosas de vejiga.

- **6 th International Conference on Olive and Olive Products. Olivebioteq.** 15-19 Octubre 2018.

Short oral presentation; Effect of modified olive pectin on proliferation of bladder cancer cells. **Bermúdez- Oria, Alejandra**; Rodríguez-Gutiérrez, Guillermo., Fernández-Prior, África, Rodríguez-Juan, Elisa., Fernández-Bolaños, Juan.

Poster: Natural bioherbicide, 3-4 dihidroxyphenylglycol from olive oil byproducts. Fernández-Prior, África., Rodríguez-Gutiérrez, Guillermo., Fernández-Bolaños, Juan., **Bermúdez- Oria, Alejandra.,** Espejo-Calvo, Juan Antonio.

- **4th Iberoamerican Congress on Biorefineries**. Jaén, Spain, from 24 to 26 October 2018,

Poster: Thermal pre-treatment in combination of anaerobic bioprocess for agro-industrial wastes utilization. Rodríguez -Gutiérrez, G., Fernández-Bolaños, J., Lama-Muñoz A., Fernández -Prior, A., **Bermúdez-Oria, A.,** Serrano, A., Borja, R., & G. Fermoso, F.

Poster: Cacao beans husk: source rich in antioxidant phenolic extracts. Hernández–Hernández, C., Morales-Sillero, A., Fernández-Bolaños, J., **Bermúdez-Oria, A**., Azpeitia-Morales, A., & Rodríguez-Gutiérrez, G.

- **Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias**. Universidad de Almería, 20 de diciembre 2018.

Comunicación oral. Extractos ricos en polisacáridos pécticos y polifenoles obtenidos del alperujo con actividad antioxidante y antiproliferativa. **Bermúdez-Oria, A.**, Rodríguez-Gutiérrez, G., Fernández-Prior, A., Girón-Calle, J., & J. Fernández-Bolaños.

Comunicaciones orales y poster presentados

- **1st International Congress on Metals in Anaerobic Biotechnologies (IMAB17)**. 4-6 Octubre 2017. Sevilla.

Short oral: Thermal treatment for olive oil wastes utilization: bioactive compounds and substrate for soil contaminated remediation. G. Rodríguez Gutiérrez**,** J. Fernández-Bolaños Guzmán, A. Lama-Muñoz. F. Rubio-Senent, A. Fernández-Prior, E. María Rodríguez Juan, A. García Borrego, **A. Bermúdez Oria**, B. Vioque-Cubero.

- **1st Congreso de Estudiantes de Doctorado en Química**. Facultad de Química 12-13 diciembre 2017.

Comunicación oral: Pectinas modificadas de aceitunas con actividad antiproliferativa en células cancerosas de vejiga.

- **6 th International Conference on Olive and Olive Products. Olivebioteq.** 15-19 Octubre 2018.

Short oral presentation; Effect of modified olive pectin on proliferation of bladder cancer cells. **Bermúdez- Oria, Alejandra**; Rodríguez-Gutiérrez, Guillermo., Fernández-Prior, África, Rodríguez-Juan, Elisa., Fernández-Bolaños, Juan.

Poster: Natural bioherbicide, 3-4 dihidroxyphenylglycol from olive oil byproducts. Fernández-Prior, África., Rodríguez-Gutiérrez, Guillermo., Fernández-Bolaños, Juan., **Bermúdez- Oria, Alejandra.,** Espejo-Calvo, Juan Antonio.

- **Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias**. Universidad de Almería, 20 de diciembre 2018.

Comunicación oral. Extractos ricos en polisacáridos pécticos y polifenoles obtenidos del alperujo con actividad antioxidante y antiproliferativa. **Bermúdez-Oria, A**., Rodríguez-Gutiérrez, G., Fernández-Prior, A., Girón-Calle, J., & J. Fernández-Bolaños.

Premios

- **Premio mejor comunicación oral**; 1st Congreso de Estudiantes de Doctorado en Química. Facultad de Química 12-13 diciembre 2017. Comunicación oral: Pectinas modificadas de aceitunas con actividad antiproliferativa en células cancerosas de vejiga.
- **Premio mejor comunicación oral**; Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias. Universidad de Almería, 20 de diciembre 2018. Comunicación oral: Extractos ricos en polisacáridos pécticos y polifenoles obtenidos del alperujo con actividad antioxidante y antiproliferativa.

ESTANCIAS

- Universidad País Vasco. Centro Lascaray Ikergunea, Vitoria. Beca Estancia breves Ministerio de España. 1 de junio 2016 hasta el 31 agosto 2016.

INFORMACIÓN ADICIONAL

- Alumna interna del departamento de Química Orgánica, facultad de Química, Universidad de Sevilla. 2011-2012.
- Prácticas de empresas. Instituto de la Grasa en el departamento de biotecnología de Alimentos Dr. Pedro García García. De julio a septiembre de 2012

- Realización del **Trabajo Fin de Grado** en IBVF (Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC) Grupo: `Bioenergética del fosfato´ Dr. Aurelio Serrano. Tutor Dr. José Román Pérez Castiñeira.
- Realización del **Trabajo Fin de Máster** en IBVF (Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC) Grupo: `Bioenergética del fosfato´ Dr. Aurelio Serrano. Tutor Dr. José Román Pérez Castiñeira.
- **Voluntariado Internacional. Dream Africa Care Foundation** (DACF). Accra, Ghana 16 julio al 6 Agosto 2018
- **Figuración:**

  Productora: Átipica film **Proyecto:** La peste 2º Temporada

  Productora: Cyan animática **Proyecto:** Video promocional, Casa museo Batlló